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EXPRESS MAIL LABEL NO. EL874429125US
DATE OF DEPOSIT: December 3, 2001

FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		5585-61760
		U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5) Not Yet Assigned 10/009376
INTERNATIONAL APPLICATION NO. PCT/GB00/02014	INTERNATIONAL FILING DATE 5 June 2000	PRIORITY DATE CLAIMED 3 June 1999

TITLE OF INVENTION
GENE THERAPY PRODUCTS

APPLICANT(S) FOR DO/EO/US
Christopher Ralph Franks, Ruggero Della Bitta, Norman James Maitland and David Jonathan Knight

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. § 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)). (Unsigned).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§ 3.28 and 3.31 and the Recordal fee of $40.00 is included.
13. ☒ A **FIRST** preliminary amendment to enter before calculating filing fee.
    ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:
    ☐ Written Opinion.
    ☒ Preliminary Examination Report.
    ☐ International Search Report.
    ☐ Copies of References Cited.


24197

EXPRESS MAIL LABEL NO. EL874429125US
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U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
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	CALCULATIONS (PTO USE ONLY)
17. ☒ The following fees are submitted:	
BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)):	
Neither International Preliminary Examination fee (37 C.F.R. § 1.482) nor International Search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,040.00**	
International Preliminary Examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
International Preliminary Examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO as an International Searching Authority **$740.00**	
International Preliminary Examination fee paid to USPTO (37 C.F.R. § 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
International Preliminary Examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)).	**$ 0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	45 - 20 =	25	x $18.00	**$ 450.00**
Independent Claims	9 - 3 =	6	x $84.00	**$ 504.00**
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	**$ 0.00**
TOTAL OF ABOVE CALCULATIONS =				**$ 0.00**
☐ Reduction of 1/2 for filing by small entity. Small entity status is claimed for this application.				**$ 1,844.00**
SUBTOTAL =				**$ 0.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 C.F.R. §§ 1.492(f)). +				**$ 0.00**
TOTAL NATIONAL FEE =				**$ 0.00**
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). **$40.00** per property. +				**$ 40.00**
TOTAL FEES ENCLOSED =				**$ 1,884.00**
			REFUND →	$
			CHARGE →	$

a. ☒ A check in the amount of $ 1,884.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees that may be required, or credit any overpayment, to Deposit Account No. 02-4550. A duplicate copy of this sheet is enclosed.

d. ☒ Please return the enclosed postcard to confirm that the items listed above have been received.

NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or § 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

KLARQUIST SPARKMAN, LLP
One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, OR 97204-2988

William D Noonan
SIGNATURE
William D. Noonan, M.D.
NAME

30,878
REGISTRATION NUMBER

cc: Docketing

WDN/SAS/GLB:clm 12/03/01 84458
Attorney Reference Number 5585-61760

Express Mail Label No. EL874429125US
Date of Deposit: December 3, 2001
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Franks *et al.*

Application No. Not Yet Assigned

Filed: Herewith

For: GENE THERAPY PRODUCTS

Examiner: Not Yet Assigned

Date: December 3, 2001

Art Unit: Not Yet Assigned

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on December 3, 2001 as U.S. Express Mail in an envelope addressed to: BOX PCT, P.O BOX 2327, ARLINGTON, VA 22202.

William D Noonan
Attorney for Applicant

BOX PCT
P.O. Box 2327
ARLINGTON, VA 22202

PRELIMINARY AMENDMENT

In the Specification:

Page 1, line 2, please insert the following:

PRIORITY CLAIM

This is a U.S. National Stage § 371 of PCT/GB00/02014, filed June 5, 2000, which was published in English under PCT Article 21(2), which claims the benefit of U.S. Provisional Application 60/137,592, filed June 3, 1999, and U.K. Application GB9912807.6, filed June 3, 1999.

By this amendment the specification has been changed to reflect prior related applications. No new matter is added by this amendment.

In the Claims:

Before calculating the filing fee, please cancel pending claims 40, 43, and 46. Please replace pending claims 1-39,41,42,44,45,47, and 48 with the following rewritten claims 1-39,41,42,44,45,47, and 48:

1. (Amended) A composition comprising a proliferatively active moiety linked to genetic or nucleic acid material which is associated with protective material.

2. (Amended) The composition of claim 1, wherein the protective material comprises a micelle-forming or complex-forming material.

3. (Amended) The composition of claim 2, wherein the complex-forming material comprises polylysine.

4. (Amended) The composition of claim 2, wherein the micelle-forming material comprises one or more phospholipids.

5. (Amended) The composition of claim 1, wherein the genetic material comprises an expression vector containing a gene encoding a protein and operably linked to a control sequence.

6. (Amended) The composition of claim 5, wherein said gene is a cytotoxic gene, a defect correction gene or an immunogene.

7. (Amended) The composition of claim 6, wherein the cytotoxic gene is for expressing an enzyme to convert a prodrug into a toxic drug.

8. (Amended) The composition of claim 7, wherein the enzyme is thymidine kinase, cytosine deaminase, cytochrome P-450 or bacterial nitroreductase.

9. (Amended) The composition of claim 5, wherein the control sequence comprises a CMV promoter.

10. (Amended) The composition of claim 5, wherein the genetic material contains an episomal maintenance sequence.

11. (Amended) The composition of claim 5, wherein the genetic material comprises two or more genes, the second and any subsequent genes each being operably linked to an internal ribosomal entry site.

12. (Amended) The composition of claim 1, wherein the genetic material comprises a plasmid construct.

13. (Amended) The composition of claim 1, wherein the nucleic acid material comprises an anti-sense sequence.

14. (Amended) The composition of claim 1, wherein the link between said agent and said moiety is intracellularly cleavable.

15. (Amended) The composition of claim 14, wherein the link is cleavable by acid hydrolysis.

16. (Amended) The composition of claim 1, wherein a target cell of the proliferatively active moiety has a high affinity receptor therefor.

17. (Amended) The composition of claim 16, wherein the proliferatively active moiety is a cytokine, growth factor, or a molecule functionally equivalent thereto.

18. (Amended) The composition of claim 17, wherein the moiety is a cytokine or a molecule functionally equivalent to a cytokine.

19. (Amended) The composition of claim 18, wherein the cytokine is an IL, a TNF, a M-CSF, an IFN, an FGF, an IGF, a TGF, a GM-CSF, an SCF, a G-CSF or an Epo.

20. (Amended) The composition of claim 19, wherein the IL is IL-2 or IL-6, the TNF is TNF-α, IFN is IFNα, IFN-β or IFN-γ and the TGF is TGFβ.

21. (Amended) The composition of claim 17, wherein the moiety is a growth factor or a molecule functionally equivalent to a growth factor.

22. (Amended) The composition of claim 21, wherein the growth factor is:
Erythropoietin (Epo);
GM-CSF;G-CSF;SCF (Stem cell factor);
Multi-CSF (also known as Interleukin-3);
M-CSF;E-CSF (or Interleukin-5);
IGF-1 (Insulin-like growth factor);
PDGF (Platelet-derived growth factor); or
TGF beta.2 (Transforming growth factor-beta.2).

23. (Amended) The composition of claim 17 wherein the cytokine or growth factor is a human cytokine or growth factor and said molecule is functionally equivalent thereto.

24. (Amended) The composition of claim 17, wherein said moiety is a recombinant human cytokine or growth factor, optionally modified by one or more amino acid alterations.

25. (Amended) The composition of claim 24, wherein the recombinant human cytokine is recombinant IL-2.

26. (Amended) The composition of claim 25, wherein the recombinant IL-2 is desala$_1$-IL-2 ser$_{125}$

27. (Amended) A composition comprising a biologically active agent which is provided with a protective material and linked to a cytokine or growth factor or to a molecule functionally equivalent thereto, the biologically active agent being selected from the group consisting of genetic material and antisense nucleotide sequences, and the cytokine or growth factor having target cells capable of presenting a high affinity receptor therefor.

28. (Amended) The composition of claim 27, wherein the protective material comprises a micelle-forming or complex-forming material.

29. (Amended) A composition comprising first domain which comprises an IL-2 sequence functional to be recognized by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises a biologically active agent selected from the group consisting of antisense nucleotide sequences and genetic material.

30. (Amended) The composition of claim 29, wherein the protective material comprises a micelle-forming or complex-forming material.

31. (Amended) A composition comprising a proliferatively active moiety linked to a nucleotide which is associated with cationic DNA-binding material.

32. (Amended) The composition of claim 31, wherein the DNA-binding material comprises a polymer, a liposome or a dendrimer.

33. (Amended) The composition of claim 32, wherein the polymer comprises polylysine, a polylysine derivative or polyethyleneimine.

34. (Amended) The composition of claim 31, wherein the DNA-binding material forms a bridge between the active moiety and the nucleotide.

35. (Amended) The composition of claim 31, wherein the DNA-binding material forms a complex with the nucleotide.

36. (Amended) The composition of claim 31, wherein the nucleotide comprises an expression vector containing a gene encoding a protein and operably linked to a control sequence or an anti-sense sequence.

37. (Amended) The composition of claims 31, wherein the link between said agent and said moiety is intracellularly cleavable.

38. (Amended) A composition comprising a first domain which comprises an IL-2 sequence functional to be recognized by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises agene for functional ADA, the gene optionally being associated with protective material.

39. (Amended) A composition comprising a functional IL-2 linked to an expression vector comprising a gene for functional ADA.

40. Please cancel claim 40.

41. (Amended) A method for treating or inhibiting the development of a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety in a subject, comprising:

administering a therapeutically effective amount of the composition of claim 1, thereby treating or inhibiting the development of the disease or disorder in the subject.

42. (Amended) The method of claim 41, wherein the composition comprises a proliferatively active moiety having IL-2 function and the disease or disorder is an autoimmune disease, transplant rejection, graft-versus-host-disease, a retroviral disease or a lymphoproliferative disease.

43. Please cancel claim 43.

44. (Amended) A pharmaceutical composition, comprising the composition of claim 1 and a pharmaceutically acceptable diluent, excipient or carrier.

45. (Amended) A method for stimulating the proliferation of a lymphocyte, comprising:

contacting the lymphocyte with the composition of claim 1, wherein the lymphocyte internalizes the compound, thereby stimulating the proliferation of the lymphocyte.

46. Please cancel claim 46.

47. (Amended) A composition comprising a moiety which is proliferatively active linked to encapsulated or complexed nucleic acid material selected from the group consisting of expression vectors and anti-sense sequences.

48. (Amended) A composition comprising a moiety having M-CSF, SCF or GM-CSF function linked to a functional acid sphingomyelinase gene.

REMARKS

Claims 40, 43, and 46 are cancelled herein. Claims 1-39, 41, 42, 44, 45, 47, and 48 are amended herein. Claims 1-39, 42, 44, and 47-48 are amended to correct form. Support for the amending language of claim 41 can be found throughout the specification, for example on page 14, lines 12-16; page 15, line 17 through page 21, line 37; page 25, line 18 through page 26, line 12; and in original claim 46. Support for the amending language of claim 45 can be found throughout the specification, for example in Fig. 1; on page 11, line 36; on page 15, lines 1-4; and on page 29, line 11 through page 30, line 4.

No new matter has been added by this amendment. Examination of the subject application is respectfully requested.

CONCLUSION

If any minor matters need to be addressed, the Examiner is invited to contact the undersigned.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

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Marked-up Version of Amended Claims and Specification
Pursuant to 37 C.F.R. §§ 1.121(b)-(c)

Page 1, line 2, please insert the following:

--PRIORITY CLAIM

This is a U.S. National Stage § 371 of PCT/GB00/02014, filed June 5, 2000, which was published in English under PCT Article 21(2), which claims the benefit of U.S. Provisional Application 60/137,592, filed June 3, 1999, and U.K. Application GB9912807.6, filed June 3, 1999.--

In the Claims:

1. (Amended) A [product] composition comprising a proliferatively active moiety linked to genetic or nucleic acid material which is associated with protective material.

2. (Amended) [A product] The composition of claim 1, wherein the protective material comprises a micelle-forming or complex-forming material.

3. (Amended) [A product] The composition of claim 2, wherein the complex-forming material comprises polylysine.

4. (Amended) [A product] The composition of claim 2, wherein the micelle-forming material comprises one or more phospholipids.

5. (Amended) [A product] The composition of [any of claims] claim 1 [to 4], wherein the genetic material comprises an expression vector containing a gene encoding a protein and operably linked to a control sequence.

6. (Amended) [A product] The composition of claim 5, wherein said gene is a cytotoxic gene, a defect correction gene or an immunogene.

7. (Amended) [A product] The composition of claim 6, wherein the cytotoxic gene is for expressing an enzyme to convert a prodrug into a toxic drug.

8. (Amended) [A product] The composition of claim 7, wherein the enzyme is thymidine kinase, cytosine deaminase, cytochrome P-450 or bacterial nitroreductase.

9. (Amended) [A product] The composition of [any of claims] claim 5 [to 8], wherein the control sequence comprises a CMV promoter.

10. (Amended) [A product] The composition of [any of claims] claim 5 [to 9], wherein the genetic material contains an episomal maintenance sequence.

11. (Amended) [A product] The composition of [any of claims] claim 5 [to 10], wherein the genetic material comprises two or more genes, the second and any subsequent genes each being operably linked to an internal ribosomal entry site.

12. (Amended) [A product] The composition of [any of claims] claim 1 [to 11], wherein the genetic material comprises a plasmid construct.

13. (Amended) [A product] The composition of [any of claims] claim 1 [to 4], wherein the nucleic acid material comprises an anti-sense sequence.

14. (Amended) [A product] The composition of [any of claims] claim 1 [to 13], wherein the link between said agent and said moiety is intracellularly cleavable.

15. (Amended) [A product] The composition of claim 14, wherein the link is cleavable by acid hydrolysis.

16. (Amended) [A product] The composition of [any of claims] claim 1 [to 15], wherein a target [cells] cell of the proliferatively active moiety [have] has a high affinity [receptors] receptor therefor.

17. (Amended) [A product] The composition of claim 16, wherein the proliferatively active moiety is a cytokine, [or] a growth factor, or a molecule functionally equivalent thereto.

18. (Amended) [A product] The composition of claim 17, wherein the moiety is a cytokine or a molecule functionally equivalent to a cytokine.

19. (Amended) [A product] The composition of claim 18, wherein the cytokine is an IL, a TNF, [and] a M-CSF, an IFN, an FGF, an IGF, a TGF, a GM-CSF, an SCF, a G-CSF or an Epo.

20. (Amended) [A product] The composition of claim 19, wherein the IL is IL-2 or IL-6, the TNF is TNF-α, IFN is IFNα, IFN-β or IFN-γ and the TGF is TGFβ.

21. (Amended) [A product] The composition of claim 17, wherein the moiety is a growth factor or a molecule functionally equivalent to a growth factor.

22. (Amended) [A product] The composition of claim 21, wherein the growth factor is:

Erythropoietin (Epo);
GM-CSF;G-CSF;SCF (Stem cell factor);
Multi-CSF (also known as Interleukin-3);
M-CSF;E-CSF (or Interleukin-5);
IGF-1 (Insulin-like growth factor);
PDGF (Platelet-derived growth factor); or
TGF beta.2 (Transforming growth factor-beta.2).

23. (Amended) [A product] The composition of claim 17 wherein the cytokine or growth factor is a human cytokine or growth factor and said molecule is functionally equivalent thereto.

24. (Amended) [A product] The composition of [any of claims] claim 17 [to 22], wherein said moiety is a recombinant human cytokine or growth factor, optionally modified by one or more amino acid alterations.

25. (Amended) [A product] The composition of claim 24, wherein the recombinant human cytokine is recombinant IL-2.

26. (Amended) [A product] The composition of claim 25, wherein the recombinant IL-2 is $desala_1$-IL-2 ser_{125}

27. (Amended) A [product] composition comprising a biologically active agent which is provided with a protective material and linked to a cytokine or growth factor or to a molecule functionally equivalent thereto, the biologically active agent being selected from the group consisting of genetic material and antisense nucleotide sequences, and the cytokine or growth factor having target cells capable of presenting a high affinity receptor therefor.

28. (Amended) [A product] The composition of claim 27,[which further includes the feature(s) recited in one or more of claims 2 to 10, 19, 20 or 22 to 26] wherein the protective material comprises a micelle-forming or complex-forming material.

29. (Amended) A [product] composition comprising first domain which comprises an IL-2 sequence functional to be recognized by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises a biologically active agent selected from the group consisting of antisense nucleotide sequences and genetic material.

30. (Amended) [A product] The composition of [any of] claim 29, [which further includes the feature(s) recited in one or more of claims2 to 12] wherein the protective material comprises a micelle-forming or complex-forming material.

31. (Amended) A [product] composition comprising a proliferatively active moiety linked to a nucleotide which is associated with cationic DNA-binding material.

32. (Amended) [A product] The composition of claim 31, wherein the DNA-binding material comprises a polymer, a liposome or a dendrimer.

33. (Amended) [A product] The composition of claim 32, wherein the polymer comprises polylysine, a polylysine derivative or polyethyleneimine.

34. (Amended) [A product] The composition of [any of claims] claim 31 [to 33], wherein the DNA-binding material forms a bridge between the active moiety and the nucleotide.

35. (Amended) [A product] The composition of [any of claims] claim 31 [to 34], wherein the DNA-binding material forms a complex with the nucleotide.

36. (Amended) [A product] The composition of [any of claims] claim 31 [to 35], wherein the nucleotide comprises [genetic material as defined in any of claims 5 to 12] an expression vector containing a gene encoding a protein and operably linked to a control sequence or an anti-sense sequence.

37. (Amended) [A product] The composition of [any of claims] claim 31 [to 36], [which further includes the feature(s) recited in one or more of claims 14 to 26] wherein the link between said agent and said moiety is intracellularly cleavable.

38. (Amended) A [product] composition comprising a first domain which comprises an IL-2 sequence functional to be recognized by high affinity IL-2 receptors and to promote

proliferation linked to a second domain which comprises agene for functional ADA, the gene optionally being associated with protective material.

39. (Amended) A [product] composition comprising a functional IL-2 linked to an expression vector comprising a gene for functional ADA.

40. (Cancelled) [A product of any of claims 1 to 39 for use as a pharmaceutical.]

41. (Amended) [The use of a product of any of claims 1 to 40 for the manufacture of a medicament] A method for treating [by therapy or prophylaxis] or inhibiting the development of a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety in a subject, comprising:

administering a therapeutically effective amount of the composition of claim 1, thereby treating or inhibiting the development of the disease or disorder in the subject.

42. (Amended) The [use] method of claim 41, wherein the [product] composition comprises a proliferatively active moiety having IL-2 function and the disease or disorder is an autoimmune disease, transplant rejection, graft-versus-host-disease, a retroviral disease or a lymphoproliferative disease.

43. (Cancelled) [A pharmaceutical formulation, comprising a product of any of claims 1 to 40 formulated for pharmaceutical use.]

44. (Amended) A pharmaceutical composition, comprising [a product] the composition of [any of claims] claim 1 [to 40] and a pharmaceutically acceptable diluent, excipient or carrier.

45. (Amended) [The use of a product of any of claims 1 to 40 for the manufacture of a medicament for internalizing the biologically active agent into a cell having a high affinity receptor for the proliferatively active moiety, cytokine or growth factor of the product and

optionally] A method for stimulating [lymphocyte] the proliferation of a lymphocyte, comprising:

contacting the lymphocyte with the composition of claim 1, wherein the lymphocyte internalizes the compound, thereby stimulating the proliferation of the lymphocyte.

46. (Cancelled) [A method of treating by therapy or prophylaxis a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety, comprising administering to a patient an effective amount of a product of any of claims 1 to 40, which product includes a proliferatively active moiety having high affinity for said receptor.]

47. (Amended) A [product] composition comprising a moiety which is proliferatively active linked to encapsulated or complexed nucleic acid material selected from the group consisting of expression vectors and anti-sense sequences.

48. (Amended) A [product]composition comprising a moiety having M-CSF, SCF or GM-CSF function linked to a functional acid sphingomyelinase gene.

GENE THERAPY PRODUCTS

FIELD OF THE INVENTION

The present invention relates to novel products which use a proliferatively active moiety as a vector to target nucleic acid material to proliferating cells and to induce proliferation of the target cells. It also relates to products which use a moiety having a high affinity receptor binding activity as vectors for delivering genetic material to selected cells. More particularly, the invention relates to novel compositions of matter for use in delivering genetic materials to cells, in gene therapy.

BACKGROUND OF THE INVENTION

Proliferation, differentiation and functional activity of most cells, particularly haematopoietic cells and cells of the immune system, are regulated by proteins called cytokines and growth factors. Separation of the two groups is difficult, due to several overlapping mechanisms and effects on target cells. For example, the cytokine Interleukin-2 is also known as T-cell Growth Factor. Cytokines and growth factors are both peptide hormones.

More specifically, cytokines regulate the functional status of their target cells (i.e. they can stimulate or suppress both quantitatively and qualitatively), whilst growth factors are more focused on promotion, regulation and maintenance of proliferation and differentation, and the survival of their target cell lineages.

Both cytokines and growth factors recognise specific membrane receptors on their target cells, which are unique for that particular cytokine or growth factor. Each receptor, in turn, can express a dynamic avidity towards its specific cytokine or growth factor, based on physiological and/or pathological conditions. These receptors can be categorised as low, medium, or high affinity. Most important of all, high affinity receptors only recognise, capture, and internalise their related cytokine or growth factor. These receptors and their ligands are discussed in more detail below with reference to cytokines.

Cytokines are a group of molecules, other than antibodies, which are produced by lymphocytes and are involved in signalling between cells of the immune system, for the purpose of stimulating or suppressing cell function. Cytokine activity is often mediated by specific receptors expressed on target cells. Cytokines are glycosylated or non-glycosylated polypeptides and can be secreted by both T-cells and B-cells, though T-cells are assumed to be the major source in cell-mediated responses. Complications in the study of cytokines have arisen from the fact that *in vivo* no cytokine ever operates in isolation. This is illustrated by the observation that many cytokine actions are synergistic. Important cytokines include interleukins (ILs), tumour necrosis factors (TNFs) and interferons (IFNs). In addition, various colony stimulating factors (CSFs) are secreted by myeloerythroid cells.

Receptors for numerous cytokines have now been cloned, and their structures (amino acid sequences) analysed. As a result, it is possible to group many of these into super families, based on common homology regions in their primary structures. For the purpose of this invention, the main super families recognised are cytokine receptor super family (CKR-SF), sometimes called haemopoietic receptor super family, and the interferon receptor super family (IFNR-SF), also termed cytokine receptor super family Type II (Ref 1). The term "super family" should be used only to describe proteins with amino acid sequence homology of 50% or less. Proteins, with amino acid sequence homology of greater than 50%, are designated by the term "family".

Many cytokine and growth factor receptors have combinations of different domains or repeats. A domain is a sequence or segment of a protein which forms a discrete structural unit, able to capture and/or convert specific signals. For the purpose of this invention, the domains of interest are the extra-cellular regions (those located at the surface of a given target cell-lineage). Studies focussing on receptor binding have revealed the existence of more than one binding affinity for several members of the CKR-SF (or haematopoietic receptor super family). Typically, these sites have low (e.g. 1-10nM) or high (e.g. at least 1pM and more usually 10-100pM) affinity to a given ligand (cytokine or growth factor). For most of these receptor complexes, additional sub-units have been identified which are required for high affinity receptor expression. These sub-units (also referred to as affinity convertors or convertor chains) are often expressed on the cell surface after a given activatory or inhibitory stimulus is applied through a receptor ligand. This results in an amplification of effects, but only in those cells bearing the high affinity receptor and not in resting cells (which usually bear the low-active receptor complexes), and is the physiological basis of any paracrine stimulation/inhibition, in the absence of any involvement of district/regional/systemic networks.

Thus, to mediate immune responses, T-cells must change from a resting to an activated state. T-cells stimulated by foreign antigens enter a program of cellular activation leading to *de novo* synthesis of IL-2. Resting T-cells do not express high affinity receptors but these are rapidly expressed after activation. Interaction of IL-2 with its induced cellular receptors triggers cellular proliferation culminating in the emergence of effector T-cells that are required for the full expression of immune responses. Taking the example of IL-2/IL-2r complex, in many of the diseases described herein, this physiological tuning is disrupted (primarily by neoplastic transformation, secondary to viruses), or is automaintained (autoimmune reactions/diseases, transplant rejection), leading to systemic multi-organ failure.

Further information about cytokines and their receptors may be found in Callard R E, Gearing A J H, The Cytokine-Factsbook, Academic Press - Harcourt Brace & Company, Publishers, 1994, 18-25.

High affinity receptors therefore include those with an affinity constant of 10^{-10}M or less, and, more particularly, those with an affinity constant of 10^{-11}M or less. Representative high affinity receptors include those with affinity constants of between 10^{-11} and 10^{-12}M. For example, three forms of receptor for interleukin-2 (IL-2) can be distinguished on the basis of their affinity for IL-2 with IL-2 binding affinities of

10^{-11}M (high affinity), 10^{-9}M (intermediate affinity) and 10^{-8}M (low affinity) (Refs 1-4). IL-2 receptors are well described in the prior arts (Refs 5 & 6).

TNF-α has been described as having two isoform receptors with high affinity on the target cells for TNF. These target cells are macrophages and osteoclasts (Ref 7). M-CSF (macrophage colony stimulating factor) has a high affinity receptor on macrophages and osteoclasts. The high affinity receptor is a 150 Kda glycoprotein (Ref 8).

High affinity receptors have been described also for IFNs (interferons). IFN-γ has a 90 KDa glycoprotein as a high affinity receptor. A different receptor present on activated lymphocytes, macrophages, endothelial cells and fibroblasts has been recognised as the high affinity receptor of IFN-α and IFN-β (Ref 9).

In the case of FGF (fibroblastic growth factor), there is a high affinity receptor which is a 140 KDa glycoprotein on mesodermic and neuroectodermic lineage cells, such as activated fibroblasts, macrophages, endothelial cells, chondrocytes, astrocytes, glioma cells, hepatocytes, epithelial cells, neurones, ovarian cells, pituitary cells, and keratinocytes. The pharmacological properties of FGF are primarily related to angiogenesis, ovarian steroidogenesis, ostoblast activation, and nerve growth (during the foetal phase) (Ref 10).

A variant GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) from the sea lamprey, *Petromyzon marinus*, has been found to suppress growth of breast, prostate and endometrial cancer cells but not to have endocrine activity at the concentrations effective against growth of cancer cells (Refs 25-28).

Epidermal growth factor (EGF) is a 53 amino acid peptide which efficiently stimulates cell growth via a receptor mediated mechanism. It is a classical example of a tyrosine kinase/SH2 domain receptor, in which extracellular EGF binding induces receptor dimerisation, autophosphorylation and binding of downstream signalling molecules to the activated receptor via their SH2 domains (Ref 29). Since the receptors for EGF are present in multiple potentially heterodimeric forms on the surfaces of a large number of cell types, this receptor type is widely applicable.

IGF (insulin-like growth factor) has a high affinity receptor on eterotetrameric complex present in different tissues and in mammary adenocarcinoma (Ref 11).

Transforming Growth Factor β (TGFβ) is similar to IGF. TGFβ is a non-glycosylated homodymeric protein secreted by fibroblasts, epithelial cells, platelets, astrocytes, monocytes, bone cells, and glioblastoma cells. The physiological target cells are primarily fibroblasts, osteoblasts, neutrophils, hematopoietic progenitors, T/B lymphocytes, and a range of tumor cells. The cytokine interacts with a high affinity receptor, expressed by the target cells, in response to paracrine microenvironmental stimulation, located on the cell surface of

the above cells. These are type 1 or type 2 receptors (55 and 80 Kda), and are able to bind to TGFβ1, 2, and 3.

GM-CSF (granulocyte/macrophage colony stimulating factor) and SCF (stem cell factor) possess a dimeric high affinity receptor in multipotent cells in the bone marrow (Ref 12). G-CSF (granulocyte colony stimulating factor) also has a high affinity receptor present, but only in multipotent cells in the bone marrow.

EPO (erythropoietin) has a multimeric high affinity receptor present on erythroid precursors in the bone marrow.

IL-6 (interleukin-6) has an α-/β- high affinity receptor. The alpha chain binds IL-6 with low affinity and exists in a soluble form. The beta chain is a 130 KDa protein which simultaneously binds IL-6/IL-6r, becoming a trimeric complex which initiates target cell stimulation. IL-6 high affinity receptor induction, following specific stimuli, is primarily positioned on activated cells such as T/B lymphocytes, fibroblasts, myeloid precursors, neurones, keratinocytes, and hepatocytes. In addition, multiple myeloma cells produce IL-6, and express IL-6 receptors working as an autocrine cancer growth factor, inducing at the same time osteoclastogenesis (bone lytic lesions). IL-6 from stromal cells can also be involved in bone metastatic lesions through different tumour histotypes.

Gonadotropin-releasing hormone (GnRH, Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is the central regulator of the hypothalamic-pituitary-gonadal axis. GnRH analogues are used to treat sex-hormone dependent cancers of the breast, prostate and ovaries. GnRH receptor RNA is expressed in human pituitary, breast, ovary, prostate and endometruim. As described by Pályi et al (Ref 24) certain cancer cell lines have been found by previous workers to express high- and low- affinity binding sites for GnRH. GnRH is therefore a peptide hormone for which there are high affinity receptors.

WO 92/20364 describes hybrid molecules containing a first portion which is a molecule capable of decreasing cell viability (especially a cytotoxin) and a second portion which is a molecule capable of specifically binding to a cytokine receptor (especially all or a binding portion of a cytokine). The second portion targets the first portion to the cytokine receptor and is exemplified as IL-2. The IL-2 portion preferably lacks IL-2 activity because the molecules will then prevent proliferation of the target cells.

Pályi et al (Ref 19) describe gonadotropin-releasing hormone (GnRH) analogue conjugates comprising peptidic analogues of human GnRH linked through lysyl side chains and a spacer (Gly-Phe-Leu-Gly) to poly(N-vinylpyrrolidine-*co*-maleic acid). The conjugated polymer protects the peptide against proteolysis and enhances its antiproliferative effect, possibly as a result of enhanced binding of the peptide conjugates with external domains of the receptor and adjacent membrane structures and/or internalisation of receptor-conjugate complexes.

Gene therapy is the transfer of genetic material or gene function modulators to target cells of a patient for the purpose of preventing or altering a disease state, and may be for the treatment of non-genetic as well as genetic disorders. Gene transfer may be *ex vivo* or *in vivo*. *Ex vivo* techniques usually involve the genetic alterations of cells, mostly by use of viral vectors, prior to implanting these into the tissues of the living body. *In vivo* gene therapy means direct introduction of genetic material into the body, and usually suffers poor efficiency because of poor access to target tissues. Proposed vectors for gene therapy include viruses (especially retrovirus and adenovirus vectors), liposomes and receptor-mediated endocytosis, for example using DNA linked to a targeting molecule such as polylysine.

A variety of gene therapy strategies are known for cancer, including haemopoietic gene transfer, immunogene therapy, delivery of toxic genes, e.g. gene-directed enzyme-prodrug therapy (GDEPT), and correction of genetic defects. GDEPT exploits the differences in gene expression between cancer cells and their normal counterparts to increase the specificity of cell destruction. A foreign gene is introduced which encodes an enzyme capable of converting a harmless prodrug into a cytotoxic compound. The system is designed so that significant transcription of the enzyme gene occurs only in tumour cells.

For further information on gene therapy see "Textbook of Gene Therapy", K K Jain, Hogrefe & Huber Publishers, 1998 (Ref 24).

The most common candidate vectors for gene therapy are viruses. However, viral vectors are seen as potentially undesirable because of doubts raised about their safety, immunity to them and the relative difficulty of large scale culture of viral vectors. Effective alternatives to viral vectors are therefore being actively sought.

The invention of International patent application No PCT/GB98/03509, the content of which is included herein by reference, is based in one aspect on an insight that a medicament which contains an active promoter of proliferation, for example an active IL-2, can beneficially be used to deliver pharmacologically desirable species to cells whose proliferation is **not** desired. For example some medicaments of that invention control or inhibit proliferation using a molecule which contains an active promoter of proliferation. Preferred embodiments are based on an appreciation that, by using the high affinity of receptor super families, it is possible to drive drugs or genetic material, for example, into specific cell lineages which are predominantly responsible for many clinical events.

International patent application No PCT/GB98/03509, therefore, describes a class of products which comprise a proliferatively active compound, especially a cytokine or growth factor, linked to a pharmacologically active compound, for example a conventional drug or a gene. The proliferatively active moiety (or cytokine or growth factor in the preferred aspect) retains its functional activity, which can come into play once the product targets its receptor. The proliferatively active moiety binds to the receptor and is then internalised by the cell, so that each active domain of the product (the proliferatively active moiety and

the biologically active agent) can perform its respective function. It is contemplated that the two domains of the product will separate intracellularly in commercially viable products, but this is not essential and the invention is not restricted to products which are intracellularly cleavable. In preferred embodiments, the events which follow binding of the proliferatively active moiety to the receptor typically include internalisation of the product (typically a fusion compound) into the cytosol (by the endosome pathway), endosome acidification (by the proton H+ pump mechanism), and a separation of functional domains into the receptor domain, the proliferatively active domain, and the active agent domain. Since the receptor domain and the proliferatively active domain (normally a cytokine or growth factor domain) retain their functional integrity, the proliferatively active domain (cytokine or growth factor) will trigger cell activation/division through DNA interaction (G2-M phase enrichment).

As the International application teaches, therefore, these proliferatively active vectors are not only active as transporters of genes, but also promote the rearrangement of DNA, in addition to opening DNA chains in target cells. They are therefore ideal for integrating genes both *in vitro* and *in vivo*. No other system available has a comparable bi-modal activity, and without the associated risks of viruses (used currently as vectors).

PCT/GB98/03509 also describes and claims products comprising a biologically active agent linked to a moiety which is a peptide hormone, which has a high affinity receptor, or is a molecule functionally equivalent to the peptide hormone in relation to the high affinity receptor.

SUMMARY OF THE INVENTION

The present application relates in particular to novel products of the types described and claimed in PCT/GB98/03509. More particularly the invention provides products or compositions of matter comprising a proliferatively active moiety linked to genetic or nucleic acid material which is associated with protective material. There is considerable evidence that transported genes are more active in dividing cells and the proliferatively active moiety therefore potentiates the cells whose proliferation is caused towards the genetic or nucleic acid material. In other words the invention is concerned with products which potentially use proliferatively active moieties both as vectors for protected nucleic acid material and as mitogens to stimulate proliferation of the target cells.

The protective material serves to protect the genetic or nucleic acid material from degradation and may by way of example comprise any known protective material. Specifically, the genetic or nucleic acid material may be protected by encapsulation in a micelle, especially a liposome, or by being complexed, for example with a protective protein such as, e.g., polylysine.

Whilst the invention includes products in which genetic or nucleic acid material is protected by an associated material, the invention is not restricted to products in which the associated material is protective

or solely protective in function. It includes also products in which genetic or nucleic acid material is otherwise associated with cationic DNA-binding material.

Accordingly the invention provides products or compositions of matter comprising a proliferatively active moiety linked to a nucleotide which is associated with cationic DNA-binding material.

It is a feature of the above aspects of the invention that the nucleic acid or genetic material is linked to a proliferatively active moiety. Unlike prior art chimeric proteins containing solely the receptor-binding domain of IL-2, therefore, these products induce cellular proliferation, enabling anti-proliferative drugs to be highly effective, even at ultra-low doses in the case of proliferatively active moieties with high affinity receptors. The invention therefore enables low systemic toxicity to be achieved. An additional benefit at least in the case of IL-2 is that IL-2 induces expression of the high affinity IL-2 receptor when the relevant antigen is present.

For example a construct of IL-2 and protected antisense DNA/RNA designed to block a retrovirus gene obtains the following effects:

a. in infected cells, the replicative stimulus given by the IL-2 stimulates also replication of the viral genome, resulting in stronger inhibitory activity by the antisense DNA/RNA;

b. immunostimulation of uninfected lymphocytes, with the potential benefit of increased immune surveillance.

Amongst the features of preferred embodiments are:

- the product is a combination of existing moieties (or of moieties functionally equivalent thereto), each of which retains its function and, optionally, its entire structure (except at any covalent linkage site to the other moiety)
- the product can be administered at exceedingly low dosages, so that little or no systemic toxicity results.
- the growth factor/cytokine stimulates the target system and the nucleic acid moiety induces a therapeutic effect
- biodistribution is predictable and good
- targeting is good

- immunogenicity is low.

An overview of the invention is shown in Fig. 1, which for convenience illustrates the invention with reference to a product in which the proliferatively active moiety is an IL-2. As shown in Fig. 1, the IL-2 is linked to a protected (in this case polylysine complexed) expression vector (in this case a plasmid), suitably by attaching to the IL-2 a linker having a functional group reactive with the polylysine as well as a functional group reactive with the IL-2, and then reacting the linker with the polylysine before or after the latter is combined with the plasmid (expression vector).

The resultant product is administered *in vivo* or *in vitro* and the IL-2 (proliferatively active moiety) serves as a vector to direct the product to cells presenting IL-2 receptors, especially high affinity receptors. The product is internalised, after which the IL-2-polylysine link is cleaved (e.g. hydrolysed), allowing the IL-2 to promote division and the expression vector to migrate to the nucleus. Less preferred products of the invention have a proliferatively active moiety which is not internalised by its receptor, in which case the link with the remainder of the product is normally cleaved extracellularly and the expression vector (or other nucleotide) is internalised.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an overview of the invention, illustrating the structure and function of products of the invention with reference to an IL-2-Expression Vector construct;

Figures 2, 3 and 4 are diagrammatic illustrations (linearised circles) of plasmid constructs of the invention;

Figure 5 is an SDS-PAGE gel run using the product of a reaction between Aldesleukin and LC-SPDP;

Figure 6 illustrates a 96 well plate as used in a growth assay;

Figure 7 is a data chart of cell densities resulting from the incubation of HT-2 cells with modified Aldesleukin; and

Figure 8 is a data chart showing growth responses of HT-2 cells to various IL-2 compositions.

Figures 9a, b and c are data charts showing the results of a toxicity assay in which serial dilutions of 25k PEI were added to HT-2 cells cultured at 1.0, 0.5 and 0.2ng/ml RhIL-2 and cell viability assayed for three days.

DESCRIPTION OF PREFERRED EMBODIMENTS

The products of the invention will now be described in more detail taking each component in turn:

The Associated Material

The material associated with the genetic or nucleic acid material is protective in one class of products. It may form a micelle, especially a liposome.

The liposome or micelle may encapsulate the nucleic acid material or it may be positively charged and hold the nucleic acid on its surface (a so-called lipoplex). Liposomes are formed by phospholipids and similar amphipathic lipids, and are commercially available. Cholesterol is frequently included in liposome formulations. A review of cationic liposomes as vectors for gene transfer may be found in Ref 38. An alternative class of encapsulating composition comprises artificial viral envelopes (see Ref 39).

One class of protective materials, therefore, comprises complexing materials and includes not only cationic liposomes but also other cationic materials, especially polymers. As suitable polymers there may be mentioned polylysine (especially poly-D-lysine), polylysine derivatives (eg phospholipid derivatives of, in particular, poly-L-lysine) and polyethyleneimine (PEI). Other suitable complexing agents are dendrimers, especially polyamidoamine dendrimers (which are cationic).

The associated material is not protective, or exclusively protective, in all the products of the invention. For example, in a class of product falling within the invention a cationic DNA-binding moiety forms a bridge between the proliferatively active product and the nucleic acid material (nucleotide). Suitable DNA-binding moieties are cationic polymers such as polylysine or PEI. Additionally there may be cationic or other protective material.

The Nucleic Acid Material

The specific identity or function of the nucleic acid or genetic material is not critical to the invention, which is concerned primarily with methods for delivery, i.e. transfer of a nucleotide into the target cell and especially into its nucleus.

The nucleic acid material generally comprises either a therapeutic gene or an antisense nucleotide (oligo- or poly- nucleotide). Antisense nucleotides are single strand nucleotides containing sequences complementary to target mRNA or DNA in order to block the production of disease-causing proteins. Antisense therapy is often considered to be a form of gene therapy because it is modulation of gene function for therapeutic purposes. Therapeutic nucleotides are suitably phosphorothioate oligodeoxynucleotides (ODNs), as these are nuclease-resistant. Alternatively, antisense nucleotides may conveniently be protein nucleic acids (PNAs).

Antisense nucleotides are useful for the treatment of viral diseases and cancer (see Ref 25) and the invention includes the use of antisense products for such treatments as well as the treatment of neurodegenerative and cerebrovascular disorders (Ref 24).

The products of the invention may be materials comprising an expression vector for expression of a therapeutic gene. The expression vector will contain a gene encoding a protein operably linked to a control sequence. The control sequence will include a promoter and preferably an enhancer derived from, for example, immunoglobulin genes, SV40, cytomegalovirus (CMV), and a polyadenylation sequence. In one class of products, the expression vector comprises a plasmid construct (recombinant plasmid).

The expression vectors are suitably in the form of recombinant plasmids. The use of a bacterial expression cassette permits large scale preparation of the construct in a prokaryotic system. Illustrative plasmids are shown in Figures 2, 3, and 4 in linearised form. Thus, Figure 2 shows a plasmid comprising a therapeutic gene, which in this case is a cytotoxic gene, represented by a gene-directed enzyme-prodrug therapy gene shown to be a thymidine kinase gene. The therapeutic gene is operably linked to a control sequence, shown to be a promoter and specifically a CMV promoter. The plasmid will also contain a poly A/termination site, a bacterial origin and an antibiotic resistance gene.

Figure 3 shows a plasmid similar to that of Figure 3 but additionally containing an episomal maintenance sequence, which will maintain the therapeutic gene outside of the cell chromosome as an episome. This will enhance its expression and prevent cellular silencing of the therapeutic gene by insertion into heterochromatin.

The construct of Figure 4 contains two therapeutic genes, which again are represented as cytotoxic genes. The construct optionally contains an IRES (internal ribosome entry site) to ensure that the two genes are expressed simultaneously from the same promoter. The two genes may be repeat copies of the same gene or may have different functions.

For experimental purposes, it is useful to make a construct having an indicator gene (e.g. the EGFP gene which causes expression of a green marker protein) and a therapeutic gene (e.g. herpes simplex virus (HSV) thymidine kinase gene which will activate gancyclovir when expressed). Cells which have been treated for transduction with the construct can be monitored for successful gene transduction by detecting for green cells. The killing drug (gancyclovir in this case) can then be added and should target the green cells by virtue of the TK gene. The process is suitably monitored by time lapse photomicroscopy, to provide information about the efficiency of cell kill as well as about the percentage of cells in the population transduced with the genes and killed.

The gene may be any gene having a therapeutic function and, by way of non-limiting example, may be a therapeutic gene now known to those skilled in the art of gene therapy. Representative classes of genes

include immunogenes (e.g. cytokine gene therapy, DNA-based cancer vaccines), cytotoxic genes and especially genes for enzyme-prodrug therapy (e.g. genes encoding viral thymidine kinase, bacterial or other cytosine deaminase, cytochrome P-450 or bacterial nitroreductase) and defect correction genes (e.g. tumour suppressor genes and especially p53).

The invention also includes products in which the nucleic acid material has a non-specific and/or non-antisense effect, for example products which inhibit viral infection by interference with absorption, penetration or uncoating of viruses.

The Proliferatively Active Moiety

Since the invention is concerned primarily with a delivery technique for nucleic acids and not with the treatment of a specific disease, the products of the invention are not restricted as to the precise identity or function of the proliferatively active moiety. However, the proliferatively active moiety is usually a cytokine or growth factor. The cytokine may be an interleukin, for example a TNF, for example an M-CSF; an IFN, for example an FGF; an IGF; a TGF, for example a GM-CSF; an SCF; a G-GSF; or an EPO.

The cytokine is preferably a human cytokine.

The growth factor may be a haematopoietic or lymphopoietic growth factor. They are a family of glycoprotein hormones which regulate survival, proliferation, and differentiation of progenitor cells, in addition to impacting on some functional activities of mature lymphohaematological cells.

Suitable growth factors include:

Erythropoietin (Epo);
GM-CSF;
G-CSF;
SCF (Stem cell factor);
Multi-CSF (also known as Interleukin-3);
M-CSF;
E-CSF (or Interleukin-5);
IGF-1 (Insulin-like growth factor);
PDGF (Platelet-derived growth factor);
TGF beta2 (Transforming growth factor -beta2)

The proliferatively active or mitogenic moiety is internalised by target cells in a preferred class of products but in another class of products is not internalised. For example, the mitogenic moiety may be a growth factor such as an EGF or FGF which is Tyr kinase/SH2 mediated. In the case of non-internalised mitogens,

the nucleic acid material is preferably cleavable from the mitogen and/or associated with a delivery material, e.g. a liposome or DNA binding material.

Applications of FGF products include their use as antiangiogenic factors in solid cancers, and to block hyperactivation of fibroblasts in scleroderma. In particular, FGF-2 and FGF-7, both of which have high affinity receptors, have been implicated in prostate cancers.

The present invention provides products comprising a domain functional to bind to an EGF, FGF-2 or FGF-7 receptor (especially a high affinity receptor) to promote proliferation and a nucleotide, e.g. genetic material. The domain or moiety having EGF, FGF-2 or FGF-7 receptor binding function serves as a vector for directing the second domain or moiety to cells having, as the case may be, EGF receptors, FGF-2 receptors or FGF-7 receptors and especially EGF high affinity receptors, FGF-2 high affinity receptors or FGF-7 high affinity receptors. The products having FGF-2 or FGF-7 receptor binding function are useful for targeting anti-cancer drugs to breast, stomach, oesophageal and prostate tumour cells.

The products having EGF receptor binding function are useful for targeting anti-cancer drugs to most tumour types but particularly those of breast, stomach, ovarian, bladder and prostatic origin.

The two domains or moieties are suitably linked by a physiologically cleavable link which will be cleaved in the receptor-bound product. The linkage between the two domains or moieties may be covalent but is not covalent in some of the products. One class of products comprises molecules having a multi-part bridging group as described above.

Cytokines or growth factors (or proliferative agents) may be native or a mutein representing the native molecule modified by one or more amino acid alterations (deletions, additions or substitutions). Such muteins, usable in the present invention, possess the biological activity of the native protein, in the sense of having both functional affinity for the receptor (and in one class of embodiments functional affinity for the high affinity receptor) and in many cases a capability of forming, with the receptor, a product internalised by the cell presenting the receptor.

The cytokines and growth factors are preferably recombinant molecules, but may be produced by cultivating cytokine or growth factor producing cell lines, for example peripheral blood lymphocytes.

In one class of embodiments, the products comprise a nucleotide associated with protective and/or DNA-binding material linked to a molecule which is functional to have a high affinity with a cytokine or growth factor high affinity receptor, and to form a complex with such a receptor which is, in one class of products, internalised by the cell presenting the receptor. In a particular class of products, the molecule may be a native or mutein cytokine, or a fragment thereof. In another class of products, the molecule may be a native or mutein growth factor, or a fragment thereof.

Particularly preferred is the cytokine interleukin-2 (IL-2). IL-2 is a lymphokine which is produced by normal peripheral blood lymphocytes, and induces proliferation of antigen or mitogen stimulated T-cells after exposure to plant lectins, antigens, or other stimuli. IL-2 was first described by Morgan, D A., at el., Science (1976), 193: 1007-1008. Then called T-cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, it is now recognised that, in addition to its growth factor properties, it modulates a variety of functions of immune system cells in vitro and in vivo, and has been renamed interleukin-2 (IL-2).

Interleukin-2 may be made by cultivating human peripheral blood lymphocytes (PBL), as described, for example, in US Patent No. 4,401,756. As a preferred alternative, the IL-2 may be recombinant. Taniguchi, T. et al., Nature (1983), 302:305-310 and Devos, R., Nucleic Acids Research (1983), 11:4307-4323 have reported cloning the human IL-2 gene and expressing it in micro-organisms.

US Patent No. 4,518,584 describes and claims muteins of IL-2 in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced with a neutral amino acid, such as serine or alanine. An oxidation-resistant mutein of IL-2 which is biologically active may be prepared wherein each methionine residue of the protein from which the mutein is derived is replaced with a conservative amino acid such as alanine; the methionine residue(s) is/are susceptible to chloramine T or peroxide oxidation. These IL-2 muteins possess the biological activity of native IL-2. US Patents Nos. 4,530,787 and 4,569,790 disclose and claim methods for purifying recombinant native IL-2 and muteins thereof, as well as purified forms of IL-2. The aforesaid US patents are included herein by reference.

The IL-2 mutein desala$_1$-IL-2 ser$_{125}$ (Aldesleukin) is available commercially from Chiron B.V. of Amsterdam, Netherlands under the trade mark Proleukin®.

The Linkage between the Proliferatively Active Moiety and the Nucleic Acid

The nature of the linkage between the proliferatively active moiety and the nucleic acid material is not critical to the invention. In some instances a bridging group is directly linked to the two of them by covalent bonding. In some other cases, a bridging group is directly covalently bonded between the proliferatively active moiety and the associated material. The bridging group may comprise a known heterobifunctional or homobifunctional linker, or it may be formed by the interbonding of two heterobifunctional linkers. In some cases, a peptide linker is used, for example between the proliferatively active material and the envelope of a liposome (see for example Ref: 19).

The proliferatively active moiety is usually peptidic, and is in a preferred class of products covalently bonded to the nucleic acid or the associated material. The peptidic moiety may be covalently bonded to the nucleic acid or associated material via a bridging moiety which is bonded to the peptide through a primary

amine residue thereof and to the other material through a functional group thereof. For example, a polylysine or PEI complexing moiety may be linked through one of its amine groups to the bridging group, and a nucleic acid may be linked through one of its hydroxy groups to the bridging groups.

The two active domains of the molecule (the proliferatively active moiety and the nucleic acid or genetic material) are in a preferred class of molecules bonded to each other via a bridging moiety which comprises a first heterobifunctional cross-linker residue bonded to the proliferatively active moiety and to a second heterobifunctional cross-linker which in turn is bonded to the nucleic acid or associated material.

Other Features

The invention also includes in another aspect a method of treating by prophylaxis or therapy a disease or disorder involving cells bearing a high affinity receptor for, in particular, a cytokine or growth factor, comprising administering to a patient an effective amount of a product comprising an agent which is biologically active when in said cells and is linked to said cytokine or growth factor; such products and preparations containing them form a further aspect of the invention.

Additionally included in the invention is a product of the invention for use as a pharmaceutical, especially in internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active agent, cytokine or growth factor of the product.

Another aspect of the invention resides in the use of a product of the invention for the manufacture of a medicament for internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active agent, cytokine or growth factor of the product and optionally for stimulating lymphocyte proliferation.

The invention will now be illustrated by way of example with reference to certain specific cytokines, growth factors, biologically active agents and diseases. Of course, the invention is not limited to these specific features.

The products of the invention preferably act only on cells presenting a high affinity receptor for the cytokine or growth factor, which are typically lymphocytes or other cells involved in the immune response. The action of a product of the invention on its target cells depends on the function of the active agent.

The molecular ratio of the active agent:nucleic acid material in the products of the invention is not critical. Thus the invention includes ratios of 1:1 or less but in some embodiments the ratio is greater than 1:1, e.g. 1:1000 or more. i.e. a plurality of active agent molecules/atoms may be bound to each proliferatively active moiety

The inventive products will now be described in more detail, by way of example, with reference to illustrative products and product classes.

Interleukin-2/antisense product

IL-2/antisense fusion products are useful for introducing specific antisense sequences (oligonucleotides) into lymphocytes bearing the IL-2 receptor.

Using cytokines (e.g. IL-2) which internalise into target cells, an antisense compound for a given tract of the HIV genome (e.g. genome coding for an envelope protein, or for the enzyme transcriptase) may be introduced specifically into the lymphocytes of a given patient, using a pharmacological strategy, such as IV administration, for example.

IL-2/antisense products may also be used to introduce antisense compounds or antioncogenes into the T-cell lineage affected by neoplastic transformation, where gene mutation, or oncogene hyperexpression is known.

Treatable Diseases

The IL-2/active compound fusion products are useful for diseases for which the lymphocyte is mainly involved in tissue damage, and the resultant development of a given disease entity.

In essence, Interleukin-2 high-affinity receptor-directed immunosuppressive therapy acts pharmacologically, but only on recently activated lymphocytes (particularly T- cells), which bear this structure on the cellular membrane. The activation signal is absent from the surface of resting T-cells and all other non-lymphoid tissues. As such, very low doses of cytokine/nucleic acids can be targeted.

Since the receptor is only transiently expressed during the brief proliferative phase, when lymphocytes respond to antigen stimuli (autologous-antigen in the case of autoimmune diseases, and heterologous-antigens in the case of transplantation), it is possible to achieve selective in vivo immunosuppression, directed solely towards activated lymphocytes (oligoclonal immunosuppression). This pharmacological action is totally different from the general immunosuppression action exerted by conventional immunosuppressive drugs.

Diseases which can benefit from this approach include autoimmune diseases, transplant rejection, HIV-infection, and lymphoproliferative diseases.

Autoimmune Diseases

Autoimmune diseases are a wide variety of disorders with a common pathogenic pathway: immune attack on target organs due to abnormal recognition of tissue antigens, and/or cellular antigens, by the immune system, particularly by T-lymphocytes (17).

This immune attack is implemented by a network of T-cell-mediated cytotoxicity, humoral autoimmune antibodies produced by B-lymphocytes, complement activation and consumption, and finally by tissue damage. The central role of the abnormal activation of the T-lymphocytes lineage in all autoimmune diseases is well recognised.

The clinical disorders under this heading and their target organs include the following:

1. Autoimmune diabetes mellitus (Type I diabetes) ---> endocrine pancreas
2. Autoimmune thyroiditis (Hashimoto and others) ---> thyroid
3. Autoimmune hepatitis (chronic active hepatitis) ---> liver
4. Rheumatoid arthritis ---> synovial/joints/viscera
5. Autoimmune Nephritis (glomerulonephritis) ---> kidney
6. Uveitis (Behcet's syndrome) ---> eye
7. Multiple sclerosis ---> CNS/PNS
8. Sjogren syndrome ---> saliva glands
9. Scleroderma ---> skin/viscera
10. Dermatopolimyositis ---> skin/muscle/viscera
11. Systemic Lupus Erythematosus(SLE)---> viscera/skin/hematopoieses/mucose
12. Autoimmune hemolytic anaemia ---> erythrocyte
13. Idiopathic thrombocytopenic purpura (ITP) ---> platelet
14. Autoimmune neutropenia ---> neutrophil
15. Vasculitis ---> vessels
16. Crohn's disease ---> bowel
17. Ulcerative colitis ---> bowel
18. Coeliac disease ---> bowel
19. Psoriasis ---> skin/joints/viscera
20. Sarcoidosis ---> lung/viscera/skin
21. Atopic syndromes.

In the majority of these pathological manifestations, there is a pathogenic lymphocyte-mediated reaction, and cytotoxicity.

The evidence to support the role of T-cells in the pathogenesis of specific disease and progression of targeted tissue damage is substantial. In most of the diseases listed above, CD4 cells (a cytotoxic subset of T-cells) are the dominant T-cell phenotype in the target tissues. T-cells express several activation markers.

Experimentally, there is evidence that autoimmune diseases improve when T-cell targeted intervention occurs, as in thoracic duct drainage, total lymphoid irradiation, and administration of Cyclosporin. Furthermore active autoimmune disease is generally less severe in AIDS patients who have CD4 cytopenia.

Most autoimmune diseases are treated by attempting to reduce the function of the immune system using immunosuppressive and anti-inflammatory drugs. This therapeutic strategy is conducted in a non-specific way, resulting at times in iatrogenic toxicity and a failure to control the overall disease process.

Lymphocytes, responsible for the acute phase of a given autoimmune attack, all bear the high-affinity IL-2 receptor on the membrane. They are antigen-activated, or cytokine-activated, lymphocytes with a high avidity for IL-2.

The parenteral administration of very-low doses of IL-2/ immunosuppressive nucleic acids enables the use of IL-2 as the vector of pharmacologically active nucleic acids to exert immunosuppression. The IL-2/immunosuppressant nucleic acid products selectively bind to, and interact with, only those cells bearing the high-affinity receptor of IL-2. This means that immune cells, responsible for tissue damage and disease progression, are inactivated selectively, potentially curing patients with chronic diseases, or reducing their relapse rate.

Recombinant proteins, for example recombinant IL-2 and other recombinant cytokines and growth factors, usually have low immunogenicity and good tissue distribution. After parenteral administration, every tissue compartment in the body is exposed, including all lymphocytes (circulating lymphocytes, lymphocytes in the tissues, and lymphocytes in the lymph nodes).

Transplantation

The acute or chronic rejection of a transplanted organ is related to heterologous antigens (antigen specific to the donor transplanted organ(s)) presenting to host T-cells. Following antigen presentation and recognition, immune cells enter into a proliferative phase, during which the high-affinity receptor for IL-2 is expressed. This leads to activation of the cytotoxic process, and to damage and subsequent failure of the transplanted organ.

The use of IL-2 as a vector to target immunosuppressive nucleic acids achieves longevity of transplanted organs without the associated toxicity of conventional immunosuppressive therapy (acute, delayed, and long term).

Allogeneic bone marrow transplantation (ABMT) is used to treat and cure leukemias (both lymphoid and myeloid), thalassemia, and solid tumours. The products of the invention have the potential to reduce the incidence of Graft-Versus-Host-Disease (GVHD), which is the reaction of the donor immune system against

tissue antigens of the host, without compromising the global immune-system (of graft origin). As a result, there could be a reduction in mortality rate due to GVHD (currently in excess of 40%), a reduction in infectious complications, and a positive anti-tumour effect on minimal residual disease (the so-called Graft-Versus-Leukaemia effect).

HIV-Infection

The administration of low-dose IL-2/antisense nucleotide specific to HIV-genome fraction exerts in vivo an immunostimulatory effect on HIV-negative lymphocytes bearing the high-affinity receptor. The antisense nucleotide is introduced into the cytoplasm of HIV-infected lymphocytes (CD4 cells), leading to a selective destruction of infected cells, without impacting on the normal reactive lymphocytes which are stimulated.

Lymphoproliferative diseases (Lymphoblastic leukaemia and lymphomas)

Using an IL-2/cytotoxic gene construct, it is possible to selectively kill the neoplastic lymphoid lineage expressing the high-affinity IL-2 receptor, without inducing any critical systemic toxicity on non-lymphoid compartments.

Products containing growth factors or cytokines other than IL-2 may be used in the therapies described below:

TNF-α has as its target cells macrophages and osteoclasts. TNF-α/blocking compound products may be used to insert into macrophages a blocking nucleotide (which blocks cellular function and/or kills the cell). Such products potentially provide an important tool in some pathological conditions, e.g. advanced solid cancers where macrophage hyperstimulation and activation is responsible for cachexia and tumour progression, monocyto-macrophage neoplasms (e.g. histiocytosis), transplant rejection and GVHD, autoimmunity, and neurological degenerative diseases (the TNF receptor in its extracellular domain is similar to nerve Growth Factor receptor).

M-CSF (macrophage colony stimulating factor) may be used to form products containing blocking nucleotides, useful for treating the same classes of conditions as TNF-α/blocking nucleotide products.

M-CSF is also responsible for microglial proliferation in the CNS. Other potential applications are therefore in some degenerative diseases of the CNS, such as Alzheimer's syndrome, and in bone diseases.

The IFN/nucleic acid products (IFN-α, -β or -γ) may be used to modify the function of activated lymphocytes, macrophages, endothelial cells and fibroblasts, or to incapacitate them in different pathological conditions, such as in HIV-infection (AIDS), and fibroblast-related diseases such as scleroderma.

Potential applications of FGF products include their use as antiangiogenic factors in solid cancers, and to block hyperactivation of fibroblasts in scleroderma. The invention enables the preparation of FGF products capable of acting as an antagonist in relation to the cell types listed earlier in this specification as having FGF high affinity receptor when activated.

IGF products may be used to treat breast cancer. Due to the presence of the high affinity receptor in CNS (neuroglia), it could also be used in some degenerative neurological disorders.

TGFβ products have applications similar to those of FGF and IGF constructs.

GM-CSF constructs may be used to selectively kill myeloid blasts responsible for myeloid leukemias. G-CSF products have similar pharmacological activity to the GM-CSF products, but bind to a different high-affinity receptor present only in multipotent stem cells in the bone marrow.

Epo fusion products may be used for diseases such as polycythemia and erythroleukemia, for example.

Epo/gene sequence fusion products, in which the DNA fraction is the normal gene for haemoglobin beta-chain, may be used for introducing the normal gene into the erythroid lineage in patients affected by beta-Thalassemia. In this genetic disease, the abnormal gene, coding for a non-functional haemoglobin beta-chain, is present in the erythroblastic progenitors in the bone marrow. The insertion of the normal gene, through the Erythropoietin vector, selectively into the bone marrow erythroblastic lineage, represents true in vivo gene-therapy, to potentially cure patients with this disease. The same consideration applies to another genetic haemoglobin disease: sickle cell anaemia.

IL-6 constructs may be used to block cells, having IL-6 high affinity receptors, which are involved in multiple myeloma, osteoclastic hyperactivation (metastasis to the bone), cancer-related bone lesions and osteoporosis.

<u>Some specific vector/gene combinations and related disease targets are set out below:</u>

1. Epo/functional Hb-beta-gene in Thalassemia

2. Epo/SS-wild gene is Sickle Cell Anemia (or Sickle Cell Disease)

3. GM-CSF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Phyladelphia + (Ph+) and its accellerated phase (Blastic crisis).

4. G-CSF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Phyladelphia + (Ph+) and its accellerated phase (Blastic crisis).

5. SCF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Phyladelphia + (Ph+) and its accellerated phase (Blastic crisis) and AML Ph+ (rare subform of AMLs).

6. IGF-1/P53 tumor-suppressor gene in breast adenocarcinoma

7. IGF-1/antisense HER-2Neu in breast adenocarcinoma

8. IL-2 (or) IL-3 (or) GM-CSF/functional genes in congenital immunodeficiencies (usually these immunodeficiencies are inherited as poligenic defects: severe combined immunodeficiencies, Di George's syndrome, Nezelof's syndrome, Ataxia-teleangiectasia, X-linked gammaglobulinemia)

9. IL-2/functional gene in selective deficiency of T-lymphocyte function such as inherited purine nucleoside phosphorylase deficiency (PNP syndrome)

10. M-CSF/functional gene in congenital macrophage enzymatic monogenic deficiencies usually present in lysosomal storage diseases. Lysosomal storage diseases include most of the lipid storage disorders, the mucopolysaccharidoses and glycoprotein storage diseases which are characterised by mono-enzymatic defects (beta-galactosidase, beta-glucocerebrosidase deficiency in Gaucher's disease, alpha-fucosidase deficiency in Fucosidosis, ceramidase deficiency in Farber's disease and hexosaminidase-A deficiency in Tay-Sachs syndrome). All these serious congenital conditions may have their onset in infantile, juvenile and adult age.

M-CSF/wild gene coding for a functional enzyme selected on the basis of the specific deficiency, once integrated into macrophages and transcripted into protein, will compete with the non-functional protein and repair the defect *in vivo*.

11. M-CSF, SCF or GM-CSF/functional gene in acid sphingomyelinase deficiency in monocytes/ macrophages in Niemann-Pick disease.

The invention includes products comprising a moiety having M-CSF, SCF or GM-CSF function linked to a functional acid sphingomyclinase gene.

<u>Adenosine Deaminase (ADA) Deficiency</u>

ADA deficiency in its most severe form results in the syndrome of Severe Combined Immunodeficiency Disease (SCID), and presents as a reduction in, and abnormal function of, both T and B lymphocytes. Less severe disease is usually associated with T-cell dysfunction and a more variable loss of B-cell function. It is now recognised that ADA deficiency can result in slowly progressive immune dysfunction, which presents at birth but also in adolescents and adults.

Approximately 20% of all patients with SCID have an ADA-gene mutation. Approximately 50% of patients with autosomal recessive inherited SCID have an ADA-gene abnormality, the remainder having other inherited abnormalities. The gene for ADA is located on chromosome 20q. More than 25 single base changes within the coding region, as well as several deletion and splicing mutations leading to loss of enzymatic activity have been identified. ADA catalyzes the irreversible deamination of adenosine to inosine, and of 2-deoxyadenosine to 2-deoxyinosine.

Allogeneic bone marrow transplantation is currently the treatment of choice for ADA-deficient SCID, but an HLA-identical donor is available only for a minority of patients. An alternative therapy is the injection of PEG-ADA (daily enzyme replacement).

The ADA gene has been sequenced and the structure of the enzyme has been determined. Several patients have been treated with ADA-cDNA *ex-vivo* transduced autologous T-lymphocytes using retroviral vectors (Refs 26-36). Results have been poor and the major barrier to effective gene therapy remains the low efficiency of stem cell transduction with retroviral vectors.

Recombinant IL-2 has been used at low doses in SCID and ADA-SCID patients (Ref 37). Therapy with low dose rIL-2 has resulted in a marked clinical improvement as well as improved T-cell function. Futhermore IL-2 is normally used to expand T-cells *in vitro* from ADA-SCID patients before ADA-cDNA transfection with retroviral vectors.

Lymphocytes and lymphoblasts from ADA-SCID patients and ADA-SCID animal model (ADA-SCID mouse) are responsive to low dose IL-2 stimulation, both *in vitro* and *in vivo*. In other words, the IL-2 mechanism and its cascade of intracellular events (cytoplasmic internalisation following high affinity receptor binding, internalisation and T-cell proliferation) is maintained in SCID.

The invention therefore includes products comprising a first domain which comprises an IL-2 sequence functional to be recognised by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises a gene for functional ADA. The gene is usually associated with protective material as described above, e.g. polylysine. Also included in the invention is a product comprising a functional IL-2 linked to an expression vector comprising a gene for functional ADA.

Preparation

Recombinant Polynucleotides

The skilled person can readily construct a variety of clones containing functional nucleic acids. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequences of nucleic acids, are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or Current Protocols in Molecular Biology, (Ausubel, et al. (eds.), Greene Publishing and Wiley-Interscience, New York (1987).

Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

Polynucleotides containing a desired gene can be prepared by any suitable method including, for example, cloning and restriction of appropriate sequences as discussed supra, or by direct chemical synthesis by methods such as the phosphotriester method of Narang et al. Meth. Enzymol. 68: 90-99 (1979); the phosphodiester method of Brown et al., Meth. Enzymol. 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage et al., Tetra. Lett., 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20):1859-1862, e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168: and, the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Nucleic acids may be modified by site-directed mutagenesis, as is well known in the art. Native and other nucleic acids can be amplified by in vitro methods. Amplification methods include the polymerase chain

reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR). A wide variety of cloning methods, host cells, and in vitro amplification methodologies are well-known to persons of skill.

Protective Material/DNA-Binding Material

Micelles or cationic materials, for example cationic polymers, are commercially available and the use of liposomes in gene therapy is reviewed by Lasic and Templeton (Lasic D D, Templeton D S (1996) *Advanced Drug Reviews* 20:221-226).

Linking

The proliferatively active moiety and one of the nucleic acid material and protective/DNA-binding material are suitably interlinked using a multifunctional (e.g. bifunctional) linker which reacts with respective functional groups on the two components. In some embodiments the two constituent parts are linked by an intracellularly cleavable link. In other embodiments, the link is intracellularly stable.

The link may comprise a bridging group comprising the inter-bonded residues of two heterobifunctional cross-linkers, as described in our US provisional patent application filed on the same day as this application under the title "Novel Constructs". The potential benefit of this technique is that it helps avoid the preparation of dimers, by separately preparing (i) an active moiety-linker construct and (ii) a nucleic acid-linker construct or protective material- or DNA-binding material- linker construct, and then reacting together constructs (i) and (ii) to join their respective linkers together. The linkers are obviously chosen to be reactive to each other, as well as to the material forming the residue of the construct (i) or (ii).

The preparation of products comprising a polypeptide linked to another moiety is well known, as for example in the case of fusion proteins, and the skilled person will therefore require no elucidation of preparatory techniques. In general terms, suitable linkers are multifunctional, and especially bifunctional compounds capable of reacting with a polypeptide and a nucleotide.

One exemplary technique involves the use of acid-cleavable reagents for interlinking two polypeptides. Such acid-cleavable linker reagents, based on orthoester, acetal and ketal functionalities, have been described previously (Ref 18), and are bifunctional compounds whose hydrolytic rate constants increase as the pH decreases. The crosslinkers react with the proteins via heterobifunctional groups (e.g. maleimide or N-hydroxysuccinimide ester) or homobifunctional groups (e.g. bis-maleimide or bis-succinimidyl).

Three particular cross-linking agents which may be used are:

1. Disuccinimidyl suberate. This is a homo-bifunctional cross-linking reagent, containing the N hydroxy succinimide ("NHS") ester reactive group, which is reactive towards amino groups. The chain of the cross-linking reagent is non-cleavable.

2. Ethylene glycobis[succinimidyl succinate]. This too is a homo-bifunctional cross-linking reagent, containing the NHS ester reactive group, which is reactive towards amino groups. The chain of the cross-linking reagent is cleavable.

3. Succinimidyl 6-[3-(2-pyridylthio)-propionamido] hexanoate. This is a hetero-bifunctional cross-linking reagent, containing the pyridyldithio and NHS ester reactive groups, which are reactive towards sulfhydryl and amino groups. The chain of the cross-linking reagent is cleavable.

A table containing more information about these crosslinkers and other suitable candidates appears below:

NAME	ABBREVIATION	REACTIVITIES
N-(β-Maleimidopropionic acid) hydrazide (trifluoroacetic acid salt)	BMPH	Sulfhydryl / Carbonyl
Disuccinimidyl suberate	DSS	Amine / Amine
Ethylene glycolbis(succinimidylsuccinate)	EGS	Amine / Amine
Succinimidyl 6-[3'-(2-pyridyldithio)-propionamido] hexanoate	LC-SPDP	Amine / Sulfhydryl
4(4-Maleimidomethyl) cyclohexane 1-carboxyl hydrazide• ½ dioxane	M_2C_2H	Sulfhydryl / Oxidised Carbohydrate (Carbonyl)
4(4-*N*-maleimidophenyl)butyric acid hydrazide hydrochloride• ½ dioxane	MPBH	Sulfhydryl / Oxidised Carbohydrate (Carbonyl)
3-(2-pyridyldithio)propionyl Hydrazide	PDP-Hydrazide	Sulfhydryl / Oxidised Carbohydrate (Carbonyl)
N-(*p*-Maleimidophenyl) isocyanate	PMPI	Sulfhydryl / Hydroxyl
Bis(sulfosuccinimidyl)suberate	BS3	
Sulfosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido] hexanoate	Sulfo-LC-SPDP	Amine / Sulfhydryl

Products in which the proliferatively active moiety (especially cytokine or growth factor) and nucleotide are acid-cleavably linked benefit from the potential advantage that the product is cleaved in the endosome to release the nucleotide in free form.

The literature describes a number of techniques for linking substances to proteins, typically by using bifunctional linker-chelating agents. For example, the reader is referred to the textbook "Laboratory Techniques in Biochemistry and Molecular Biology", Volume 19, edited by R. H. Burdon and P. H. van Knippenberg, published by Elsevier and to Chapter 3 ("Peptide-Carrier Conjugation") of the textbook "Synthetic Polypeptides as Antigens" by M. H. V. Van Regenmortel et al, published by Elsevier (1988).

Heterobifunctional linkers suitable for conjugating cytokines and growth factors to biologically active agents are obtainable from Pierce & Warner (UK) Limited, 44 Upper Northgate Street, Chester CH1 4EF, UK, or Pierce Chemical Company, PO Box 117, Rockford, IL 61105, USA, whose literature provides further information.

Administration

The IL-2 and other cytokines or growth products may be administered parenterally, in contemplated amounts of from 10.000 - 1.000.000 International Units, and suitably by intravenous, intramuscular or subcutaneous injection e.g. (less than 1 µg to 0.1 mg of recombinant protein), to give very low plasma concentrations. For example, the plasma IL-2 concentrations may be close to the dissociation constant (KD) of the concentration of IL-2 that saturates 50% of the IL-2 high-affinity receptor isoform. Furthermore, this range of dose is generally without systemic adverse side effects.

The products of the invention may be formulated as human or veterinary pharmaceutical formulations in practice comprising a pharmaceutically acceptable diluent carrier or excipient.

The formulations may be in the form of solutions or suspensions. The formulations are suitable for parental (e.g. iv or sc) administration but, oral formulations are not excluded.

EXAMPLES

Protocols suitable for crosslinking nucleotide material with peptides, exemplified by the IL-2 mutein Aldesleukin, will now be described. The specific reaction conditions may of course be varied from those described.

Modification of Proteins Using LC-SPDP.

LC-SPDP = Succinimidyl 6-[3'(2-pyridyldithio)-propionamido]hexanoate

Materials:

A: LC-SPDP stock solutions: 20mM LC-SPDP in DMSO Note: Prepare just before use.

B: Reaction buffer: 20mM sodium phosphate, 150mM sodium chloride, 1mM EDTA, pH7.5

C: Acetate buffer: 100mM sodium acetate, 100mM sodium chloride, pH4.5

D: BioRad Micro Bio-Spin Desalting Columns

E: Dithiothreitol (DTT): 24mg/ml in 100mM sodium acetate pH4.5, 100mM sodium chloride.

F: Aldesleukin stock solution: Dissolved at 1.53mg/ml (0.1mM) in reaction buffer

Method:

1. To 9µl of Aldesleukin stock solution, add 1µl of the 20mM LC-SPDP stock solution.
2. Incubate for 30 minutes at room temperature
3. To remove the unconjugated cross-linker, the sample is applied to a BioRad Micro Bio-Spin Desalting Column (pre-equilibrated in 100mM sodium acetate, 100mM sodium chloride, pH4.5), spin and the filtrate used for the subsequent DTT treatment.
4. DTT (dissolved as above in the appropriate buffer) is added to a final concentration of 8mg/ml.
5. Incubate at room temperature for 30 minutes

6. To remove the DTT, the sample is applied to another BioRad Micro Bio-Spin Desalting Column (pre-equilibrated in conjugation buffer: 20mM sodium phosphate pH7.5, 150mM sodium chloride, 1mM EDTA), spin and use the filtrate for the final conjugation reaction (see later).

Modification of Nucleotide Material using PMPI.

PMPI = N-(p-Maleimidophenyl) isocyanate

Materials:

A: PMPI stock solution: 50mM in dry DMSO, freshly prepared before use

B: Reaction Buffer: 20mm Tris-HCl pH8.5, 100mM NaCl

C: BioRad Micro Bio-Spin desalting columns

Method:

1: Add 1µl 50mM PMPI in dry DMSO to genetic material. Mix thoroughly.

2: Incubate at room temperature for 60 minutes

3: Remove the excess unreacted cross-linking reagent using a BioRad Micro Bio-Spin desalting column, pre-equilibrated in conjugation buffer (20mM sodium phosphate pH 7.5, 150mM sodium chloride, 1mM EDTA). The filtrate is used for the final conjugation reaction (see below).

Conjugation Reaction:

The desalted LC-SPDP-activated Aldesleukin and the desalted PMPI-activated genetic material are mixed in equal proportions and incubated at room temperature for 24 hours. The conjugation reaction is then analysed by SDS-PAGE, along with samples from the activation reactions, to assess the conjugation efficiency. Conjugated product can then be purified by size-exclusion chromatography.

Example 1

Production of modified Aldesleukin

Modification of Aldesleukin was performed according to the protocol set out above, except that 7 reactions were run for 0, 10, 20, 30, 40, 50 and 60 minutes at room temperature. The reaction mixtures were desalted as per the protocol (using BioRad Micro Bio-spin P6 desalting columns, used according to the manufacturer's protocols). A 1µl sample was removed from each filtrate for analysis by SDS-PAGE. [A 12% acrylamide gel, run according to the method of Laemmli (Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970 Aug 15 227:259 680-5)]. The gel was then stained using Gelcode Blue (Pierce; used according to manufacturer's protocol).

A gel of a 60 minute run is shown in Figure 5. The picture shows the increase in apparent molecular weight after modification of Aldesleukin by LC-SPDP to illustrate that all of the Aldesleukin is covalently modified.

Example 2

Growth Assay

The column filtrates from Example 1 were resuspended to approximately 0.20 µg/ml in standard HT-2 culture medium (Iscove's Modified Dulbecco's Medium with added 10% Foetal Bovine serum, 20µM 2-mercaptoethanol) and used in growth assays according to the protocol set out below.

The assay samples were set up to contain 20ng/ml modified Aldesleukin species (1 set of samples from each reaction product), and plated out onto a 96-well plate. The final row was plated out containing only the recombinant IL-2 carried over from the stock cell culture.

(HT-2 cells are standard cells for measuring induction of proliferation of T-lymphocytes, see Watson J Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J Exp Med* 1979 Dec 1 150:6 1510-9.)

Growth Assay Protocol

A: Setting up the Assay.

1. From the same master cell stock, set up 8 x 2ml aliquots of HT-2 cells in sterile containers in Iscove's Modification of Dulbecco's Medium (plus 10% FCS, 20µM ß-mercaptoethanol, 10IU/ml recombinant human IL-2, or equivalent), at a cell density of between 20,000 and 30,000 per ml
2. To each, add the IL-2 under test to the desired concentration.
3. Plate out the cell suspensions into 96-well plates at 150µl per well as shown in Figure 6
   Grow on in an incubator for 4 days, counting the cells each day.
4. Pool the remaining cell suspensions from the sterile containers for cell counting to establish a baseline cell density (use n=3 for the counts).

B: The Cell Counting.

1. Each day, count the cells which appear to be of both relatively normal morphology (round or nearly round), and exclude trypan blue, using a haemocytometer. Note the time at which the cell counts were performed.
2. Count three wells for each test condition per day, and average the results.

Example 3

Cell Viability

The cell density in the initial cultures from Example 2 was assessed using a haemocytometer, and the viable cells assessed by Trypan blue exclusion. These were counted in triplets, as were all samples throughout the assay period. The averaged cell densities are shown in following Table1:

Average Cell Densities

	0	24	48	80	100
0 minutes	32000	49333	180000	762667	533333
10 minutes	32000	49333	176000	86667	7000
20 minutes	32000	68000	149333	50667	13333
30 minutes	32000	41333	186667	57333	10667
40 minutes	32000	80000	193333	37333	8000
50 minutes	32000	60000	185333	48000	13333
60 minutes	32000	58667	173333	69333	12000
Pos. Control	32000	44000	229333	601333	110667
Al/Filt	32000	65333	220000	118667	8000
Neg. Control	32000	30666	0	0	0

The results are presented in a data chart in Figure 7.

Comparative growth responses to various IL-2 compositions comprising Aldesleukin (Proleukin®), native human IL-2 and recombinant human IL-2 are shown in Figure 8, and indicate that any of these IL-2s is a suitable mitogenic vector.

Example 4

Transfection of pEGFP-1 into HT-2 cells

pEGFP-1 was transfected into HT-2 cells using Transfectam (Promega). The numbers of cells that expressed EGFP is shown in Table 2. The highest transfection efficiency (approximately 0.08%) was observed with a cationic lipid : DNA ratio of 4:1 using either Dfx20 or Dfx50. The number of cells that were transfected was considerably higher when cultured at 1.0ng/ml RhIL-2 compared with 0.2ng/ml RhIL-2. This may reflect the fact that these cells are proliferating more quickly and as a consequence of nuclear breakdown during the cell cycle it is easier for the plasmids to enter the nucleus and express their genes. Support for this theory comes from the observation that many of the EGFP expressing cells were found in

pairs which suggests that they may have recently divided. These results indicate that IL-2 and other proliferatively active moieties potentiate cells towards genetic or nucleic acid material.

Table 2: Number of cells which express EGFP 28 hours after transfection

0.2ng/ml RhIL-2		Ratio Tfx:DNA					
	Amount of DNA (ng)	2:1		4:1		6:1	
Tfx10	250	0	0	0	0	0	0
	500	0	0	0	0	0	0
Tfx20	250	0	0	1	0	0	0
	500	0	0	0	0	0	
Tfx50	250	1	0	1	0	0	0
	500	4	3	4	0	0	0
1.0ng/ml RhIL-2		Ratio Tfx:DNA					
	Amount of DNA (ng)	2:1		4:1		6:1	
Tfx10	250	0	0	0	0	0	3
	500	0	0	0	1	0	2
Tfx20	250	0	0	9	8	1	4
	500	0	7	10	13	0	2
Tfx50	250	32	5	14	15	21	7
	500	2	8	24	5	3	6

Example 5

Characterisation of Polyethelinimine (25k)

25k PEI, in contrast to 800k PEI, is more homogeneous in terms of polymer length and has been reported to have fewer toxic effects. Since a more homogeneous product would simplify the interpretation of crosslinked products, 25k PEI was investigated as a possible candidate cationic polymer for this study. Initially 25k PEI was run on the column to determine the size profile. Comparison with the profile of 800k PEI reveals an equally heterogeneous range of species.

A toxicity assay using serial dilutions of 25k PEI added to HT-2 cells cultured at 1.0, 0.5 and 0.2ng/ml RhIL-2 was performed and cell viability assayed for three days (see Figures 9a - c). There was a drop in cell viability at 2µg/ml 25k PEI in cells grown at 1.0 or 0.5ng/ml RhIL-2 whilst those cultured at 0.2ng/ml RhIL-2 showed a fall in viability at 1µg/ml 25k PEI. 25k PEI is slightly more toxic than 800k PEI for HT-2 cells which is contrast to the published results for other cell lines.

Example 6

Transfection of PEGFP.CAT into HT-2 cells using Polyethylenimine

An assay was performed to determine if polyethylenimine (PEI) can form complexes with the reporter plasmid which are capable of transfecting HT-2 cells. Various ratios of PEI:DNA were used to ensure

optimal DNA compaction. In addition cells were grown at 1.0ng/ml RhIL-2 for 24 hours prior to the application of the DNA:PEI complexes, before being split into two plates, one at 1.0ng/ml RhIL-2 and the other at 0.2ng/ml RhIL-2. The transfection complexes were then applied to the cells and centrifuged at 500rpm for 5 min. After one hour incubation all the cells were covered with complete media containing 1.0ng/ml RhIL-2. The number of cells expressing EGFP 20 hours after transfection are shown in Table 3.

Table 3

PEI:DNA ratio	1.0ng/ml RhIL-2		0.2ng/ml RhIL-2	
	800kD PEI	25kD PEI	800kD PEI	25kD PEI
500ng:500ng	6	30	5	10
250ng:500ng	0	152	1	66
100ng:500ng	0	0	0	0
66.6ng:500ng	0	0	0	0
50ng:500ng	0	0	0	0
10ng:500ng	0	0	0	0
100ng:500ng + chloroquinone	2	27	0	20

The following conclusions may be drawn

- The number of cells transfected increased with RhIL-2 concentration
- 25kD PEI is far more efficient at gene delivery than 800kD PEI
- 25kD PEI is 2-3 times more efficient than the commercial transfection reagent Transfectam (comparative data not shown)
- a ratio of 250ng PEI to 500ng DNA was the most efficient
- the presence of chloroquinone improved the transfection efficiency.

A second assay was performed to investigate the effect of the length of incubation of the 25kD PEI:DNA complexes before the addition of complete media (containing serum). The ratio of PEI:DNA was further investigated and the effect of chloroquinone more fully examined. The number of cells expressing EGFP 18 hours after transfection are shown below in Table 4:

Table 4

25kDPEI:DNA ratio	1 hour incubation		2 hour incubation	
	- chloroquinone	+ chloroquinone	+- chloroquinone	- chloroquinone
300ng:500ng	6	18	1	26
250ng:500ng	21	72	14	73
200ng:500ng	43	273	45	245
150ng:500ng	25	21	20	143
100ng:500ng	247	0	157	0

The following conclusions may be drawn

- 200ng of PEI with 500ng of DNA gave the highest transfection efficiency in the presence of chloroquinone

- in the absence of chloroquinone the highest transfection efficiency was with 100ng PEI in contrast to the result of the previous assay
- chloroquinone increased transfection efficiency by 3 to 6 times at higher PEI concentrations
- there was no significant increase in transfection efficiency when the incubation time was increased from 1 hour to 2 hours
- 18 hours after transfection was considered most appropriate for examination of gene expression due to the toxic effect of marker gene products.

In summary, mild centrifugation (data not shown), the use of chloroquinone and the use of 25kD PEI all helped to improve the delivery of the marker genes to HT-2 cells.

As used herein the word "comprises" is not exclusive, i.e. it indicates that the subject of the verb need not consist only of its object but may include the object of the verb and one or more additional elements. Cognate expressions are to be construed accordingly.

References

1. Smith. K.A. (1988) *Science* 240:116-1176
2. Waldmann. T.A. (1991) *J.Biol.Chem.* 266:2681-2684
3. Waldmann. T.A. (1989) *Annu.Rev.Biochem.* 58:875-911
4. Kuziel. W.A. and Greene, W.C. (1990) *J.Invest.Dennatol.* 94:275-325
5. EP 319012 (Du Pont)
6. Waldmann, T.A. (1993) *Immunol. Today* 14:264-269
7. Cerami and Beuter (1988) *Immunol. Today* 9:28
8. Suzu et al. (1992) *J.Biol.Chem.* 267:4345
9. Sato et al. (1992) *Cancer Res.* 52:444
10. Gabbianelli et al. (1990) *J.Biol.Chem.* 249:1252
11. Zumstein et al. (1987) *J.Biol.Chem.* 262:1252
12. Bussolino et al. (1989) *Nature* 337:471
13. Signore A. et al. (1987) *The Lancet*, VOL II, No. 8558
14. Signore A. et al. (1992) *Nuclear med. Comm.* 13:713-722
15. Chianelli A. et al. *Nuclear medicine Proceed. EANM* 1991:143-146
16. Bellan-harel A. et al. (1989) *J.Immunological Methods* 119:127-133
17. Godoman & Gilman The Pharmacological Basis of Therapeutics, Eighth Edition 1990
18. Neville D M et al., (1989) *J.Biol.Chem.* 264:14653-14661
19. Pályi I et al, (1999) *Proc. Natl. Acad. Sci. USA* Vol 96, pp2361-2366
20. Pályi I et al, (1995) *5th International Congress on Hormones and Cancer*, ed. Labrie J (ESCOM, Leiden, the Netherlands, p87.
21. Pályi I et al, (1996) *Ann. Oncol.* 7, 83
22. Pályi I et al, (1997) *Proc. Am. Assoc. Cancer Res.* 38, 61
23. Vincze B et al, (1997) *Proc. Am. Assoc. Cancer Res.* 38, 433
24. K K Jaine "Textbook of Gene Therapy", Hografe & Huber Publishers, 1998
25. Wagner R W et al, (1997) *Molecular Medicine Today* 3:31-38
26. Blaese R M et a. Treatment of severe combined immunodeficiency disease (SCID) due to adenosine deaminase deficiency with CD34+ selected autologous peripheral blood cells transduced with a human ADA gene. Amendment to clinical research project, Project 90-C-195, January 10, 1992. *Hum Gene Ther* 1993 Aug; 4(4):521-7.
27. Blese R M. Development of gene therapy for immunodeficiency: adenosine deaminase deficiency. *Pediatr Res* 1993 Jan;33(Suppll):S49-53; discussion S53-5.
28. Kaptein L C et al. Bone marrow gene therapy for adenosine deaminase deficiency. *Immunodeficiency* 1993;4(1-4):335-45.

29. Kawamura N et al. Elevation of serum IgE level and peripheral eosinophil count during T-lymphocyte-directed gene therapy for ADA deficiency:implication of Tc2-like cells after gene transduction procedure. *Immunol Lett* 1998 Nov;64(1):49-53/

30. Onodera M et al. Successful peripheral T-lymphocyte-directed gene transfer for a patient with severe combined immune deficiency caused by adenosine deaminase deficiency. *Blood* 1998 Jan 1;91(1):30-6.

31. Sakiyama Y. Gene therapy for adenosine deaminase deficiency. *Okkaido Igaku Zasshi* 1996 Jan;71(1):27-32.

32. Kohn D B et al. Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nat Med* 1995 Oct;1(10):1017-23.

33. Hoogerbrugge P M et al. Gene therapy for adenosine deaminase deficiency. *Br Med Bull* 1995 Jan; 51(1): 72-81.

34. Blaese R M et al. T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* 1995;270:475-480.

35. Bordignon C et al. Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* 1995;270:470-475.

36. Bordignon C et al. Transfer of the ADA gene into bone marrow cells and peripheral blood lymphocytes for the treatment of patients affected by ADA-deficient SCID. *Hum Gen Ther* 1993;4:513-20.

37. Pahwa R et al. Recombinant interleukin-2 therapy in severe combined immunodeficiency disease. *Proc Natl Acad Sci* USA 1989 Jul; 86(13): 5069-73.

38. Gao X et al. (1995) *Gene Therapy* 2:710-722

39. Philips S (1996) *Exp Opin Invest Drugs* 5:1101-1115

CLAIMS

1. A product comprising a proliferatively active moiety linked to genetic or nucleic acid material which is associated with protective material.

2. A product of claim 1, wherein the protective material comprises a micelle-forming or complex-forming material.

3. A product of claim 2, wherein the complex-forming material comprises polylysine.

4. A product of claim 2, wherein the micelle-forming material comprises one or more phospholipids.

5. A product of any of claims 1 to 4, wherein the genetic material comprises an expression vector containing a gene encoding a protein and operably linked to a control sequence.

6. A product of claim 5, wherein said gene is a cytotoxic gene, a defect correction gene or an immunogene.

7. A product of claim 6, wherein the cytotoxic gene is for expressing an enzyme to convert a prodrug into a toxic drug.

8. A product of claim 7, wherein the enzyme is thymidine kinase, cytosine deaminase, cytochrome P-450 or bacterial nitroreductase.

9. A product of any of claims 5 to 8, wherein the control sequence comprises a CMV promoter.

10. A product of any of claims 5 to 9, wherein the genetic material contains an episomal maintenance sequence.

11. A product of any of claims 5 to 10, wherein the genetic material comprises two or more genes, the second and any subsequent genes each being operably linked to an internal ribosomal entry site.

12. A product of any of claims 1 to 11, wherein the genetic material comprises a plasmid construct.

13. A product of any of claims 1 to 4, wherein the nucleic acid material comprises an anti-sense sequence.

14. A product of any of claims 1 to 13, wherein the link between said agent and said moiety is intracellularly cleavable.

15. A product of claim 14, wherein the link is cleavable by acid hydrolysis.

16. A product of any of claims 1 to 15, wherein target cells of the proliferatively active moiety have high affinity receptors therefor.

17. A product of claim 16, wherein the proliferatively active moiety is a cytokine or growth factor or a molecule functionally equivalent thereto.

18. A product of claim 17, wherein the moiety is a cytokine or a molecule functionally equivalent to a cytokine.

19. A product of claim 18, wherein the cytokine is an IL, a TNF, and M-CSF, an IFN, an FGF, an IGF, a TGF, a GM-CSF, an SCF, a G-CSF or an Epo.

20. A product of claim 19, wherein the IL is IL-2 or IL-6, the TNF is TNF-α, IFN is IFNα, IFN-β or IFN-γ and the TGF is TGFβ.

21. A product of claim 17, wherein the moiety is a growth factor or a molecule functionally equivalent to a growth factor.

22. A product of claim 21, wherein the growth factor is:
Erythropoietin (Epo);
GM-CSF;
G-CSF;
SCF (Stem cell factor);
Multi-CSF (also known as Interleukin-3);
M-CSF;
E-CSF (or Interleukin-5);
IGF-1 (Insulin-like growth factor);
PDGF (Platelet-derived growth factor);
TGF beta2 (Transforming growth factor-beta2).

23. A product of claim 17 wherein the cytokine or growth factor is a human cytokine or growth factor and said molecule is functionally equivalent thereto.

24. A product of any of claims 17 to 22, wherein said moiety is a recombinant human cytokine or growth factor, optionally modified by one or more amino acid alterations.

25. A product of claim 24, wherein the recombinant human cytokine is recombinant IL-2.

26. A product of claim 25, wherein the recombinant IL-2 is desala$_1$-IL-2 ser$_{125}$

27. A product comprising a biologically active agent which is provided with a protective material and linked to a cytokine or growth factor or to a molecule functionally equivalent thereto, the biologically active agent being selected from the group consisting of genetic material and antisense nucleotide sequences, and the cytokine or growth factor having target cells capable of presenting a high affinity receptor therefor.

28. A product of claim 27, which further includes the feature(s) recited in one or more of claims 2 to 10, 19, 20 or 22 to 26.

29. A product comprising first domain which comprises an IL-2 sequence functional to be recognised by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises a biologically active agent selected from the group consisting of antisense nucleotide sequences and genetic material.

30. A product of any of claim 29 which further includes the feature(s) recited in one or more of claims 2 to 12.

31. A product comprising a proliferatively active moiety linked to a nucleotide which is associated with cationic DNA-binding material.

32. A product of claim 31, wherein the DNA-binding material comprises a polymer, a liposome or a dendrimer.

33. A product of claim 32, wherein the polymer comprises polylysine, a polylysine derivative or polyethyleneimine.

34. A product of any of claims 31 to 33, wherein the DNA-binding material forms a bridge between the active moiety and the nucleotide.

35. A product of any of claims 31 to 34, wherein the DNA-binding material forms a complex with the nucleotide.

36. A product of any of claims 31 to 35, wherein the nucleotide comprises genetic material as defined in any of claims 5 to 12 or an anti-sense sequence.

37. A product of any of claims 31 to 36, which further includes the feature(s) recited in one or more of claims 14 to 26.

38. A product comprising a first domain which comprises an IL-2 sequence functional to be recognised by high affinity IL-2 receptors and to promote proliferation linked to a second domain which comprises a gene for functional ADA, the gene optionally being associated with protective material.

39. A product comprising a functional IL-2 linked to an expression vector comprising a gene for functional ADA.

40. A product of any of claims 1 to 39 for use as a pharmaceutical.

41. The use of a product of any of claims 1 to 40 for the manufacture of a medicament for treating by therapy or prophylaxis a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety.

42. The use of claim 41, wherein the product comprises a proliferatively active moiety having IL-2 function and the disease or disorder is an autoimmune disease, transplant rejection, graft-versus-host-disease, a retroviral disease or a lymphoproliferative disease.

43. A pharmaceutical formulation, comprising a product of any of claims 1 to 40 formulated for pharmaceutical use.

44. A pharmaceutical composition, comprising a product of any of claims 1 to 40 and a pharmaceutically acceptable diluent, excipient or carrier.

45. The use of a product of any of claims 1 to 40 for the manufacture of a medicament for internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active moiety, cytokine or growth factor of the product and optionally for stimulating lymphocyte proliferation.

46 A method of treating by therapy or prophylaxis a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety, comprising administering to a patient an effective amount of a product of any of claims 1 to 40, which product includes a proliferatively active moiety having high affinity for said receptor.

47. A product comprising a moiety which is proliferatively active linked to encapsulated or complexed nucleic acid material selected from the group consisting of expression vectors and anti-sense sequences.

48. A product comprising a moiety having M-CSF, SCF or GM-CSF function linked to a functional acid sphingomyelinase gene.

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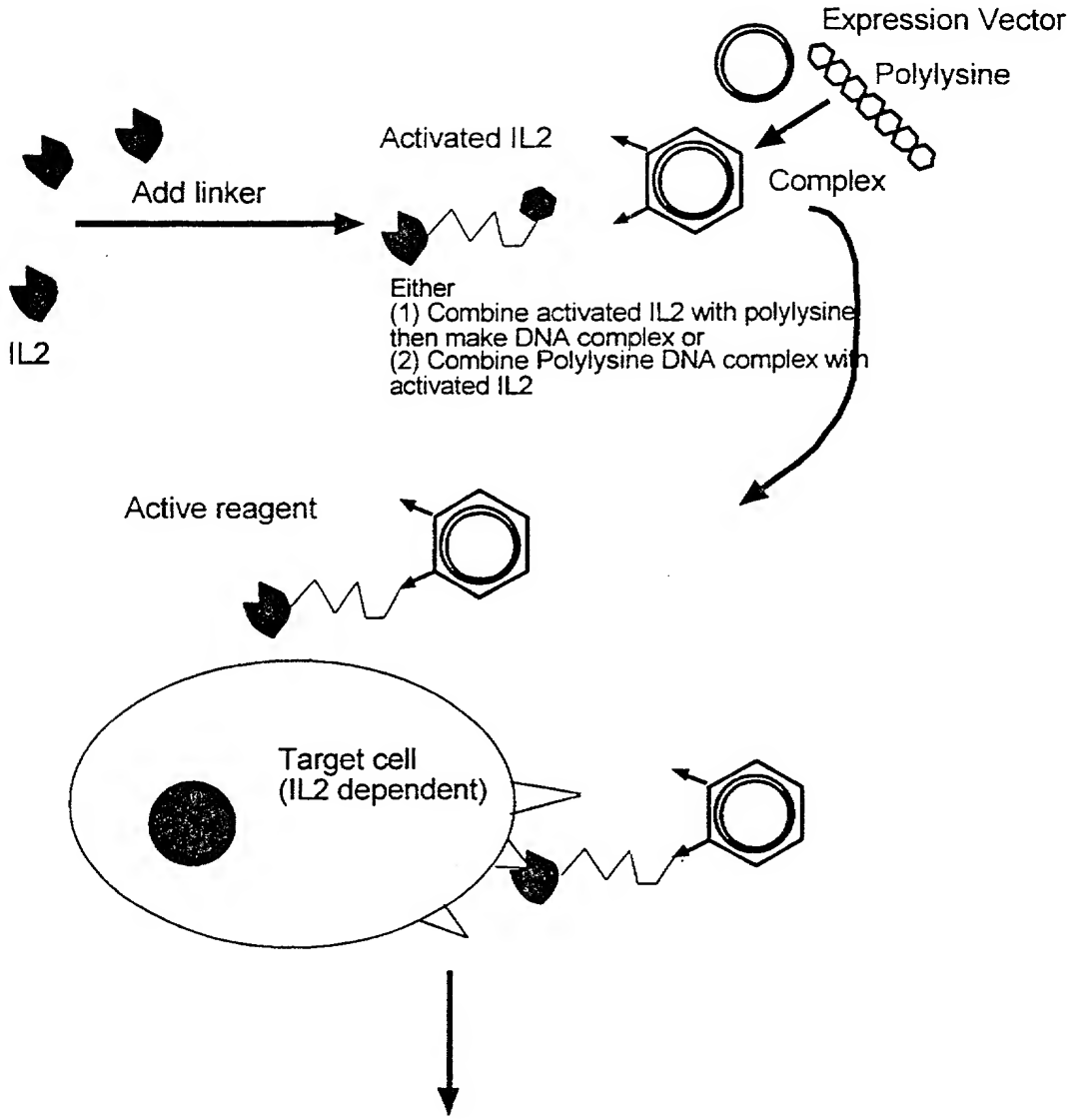
(54) Title: GENE THERAPY PRODUCTS

(57) Abstract: A product comprising a proliferatively active moiety linked to genetic or nucleic acid material which is associated with protective material. The proliferatively active moiety is typically a cytokine or growth factor. The protective material may form a micelle, especially a liposome, which may encapsulate the nucleic acid material or be positively charged and hold the nucleic acid on its surface (a so-called lipoplex). One class of protective materials, therefore, comprises complexing materials and includes not only cationic liposomes but also other cationic materials, especially polymers. As suitable polymers there may be mentioned polylysine (especially poly-D-lysine), polylysine derivatives (e.g. phospholipid derivatives of, in particular, poly-L-lysine) and polyethyleneimine (PEI). Other suitable complexing agents are dendrimers, especially polyamidoamine dendrimers (which are cationic). The genetic or nucleic acid material may be a cytotoxic gene, a defect correction gene or an immunogene. Suitable cytotoxic genes are for expressing an enzyme to convert a prodrug into a toxic drug.



1. Cells with high affinity IL2 receptors take up many IL2 molecules in a receptor mediated mechanism of response (and the linked molecule)
2. Internalised DNA polylysine complex migrates to nucleus, avoiding lysosomal trap and expresses the linked gene. The expression of the linked gene is enhanced by the IL2 induced replicative response

Fig 1

Plasmid constructs (linearised circles):

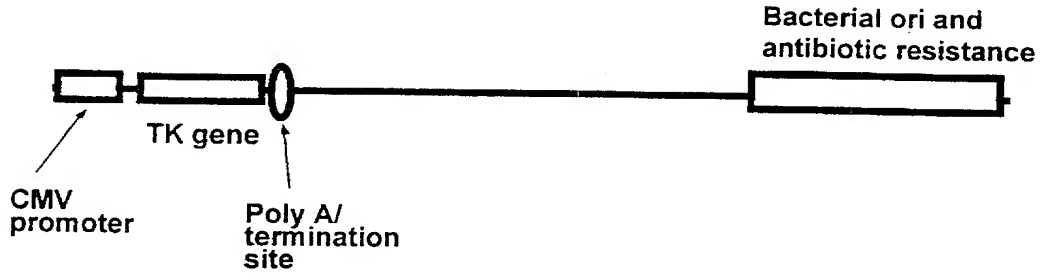


Fig 2

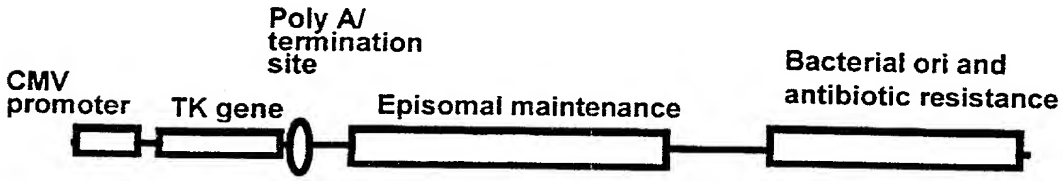


Fig 3

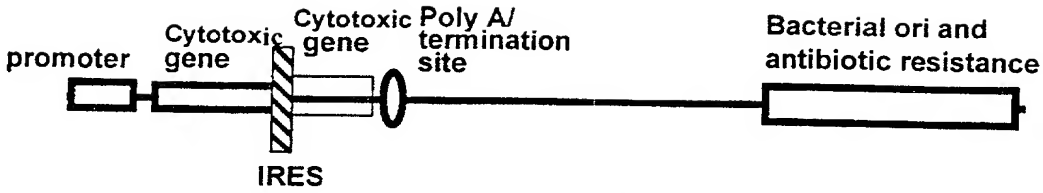
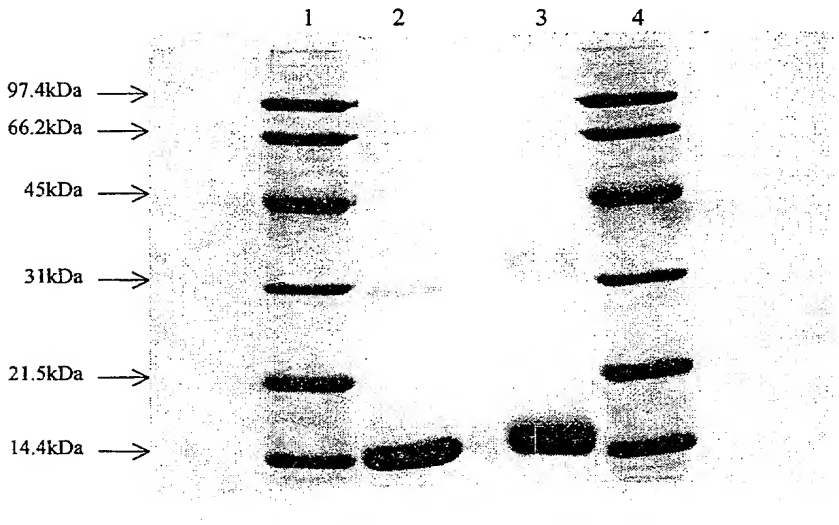


Fig 4

Activation of Proleukin using

N-succinimidyl 6-[3'-(2-pyridyldithio)propionamido] hexanoate (LC-SPDP)



Lanes as marked on the image above contain the following:

1: Molecular Weight Markers

2: Control Reaction (DMSO only)

3: LC-SPDP Activation reaction (2mM LC-SPDP for 40 minutes)

4: Molecular Weight Markers

Fig 5

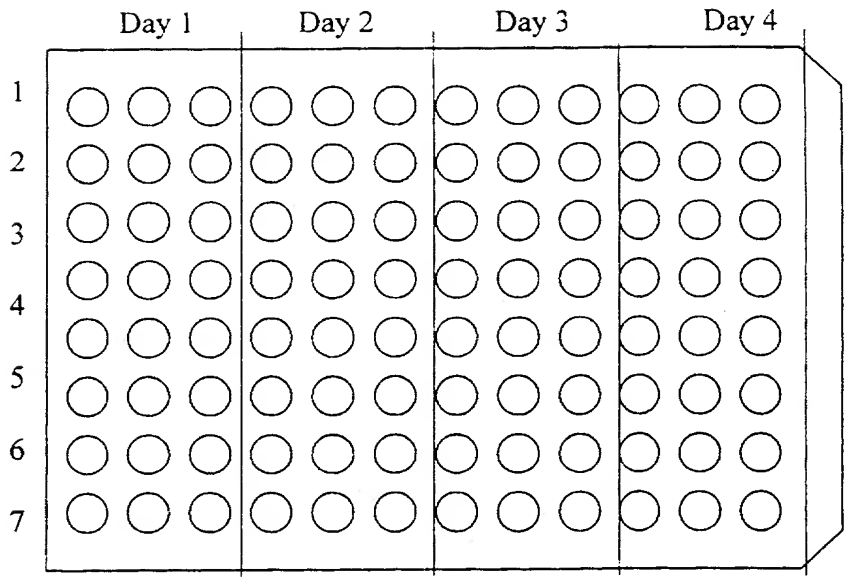


Fig 6

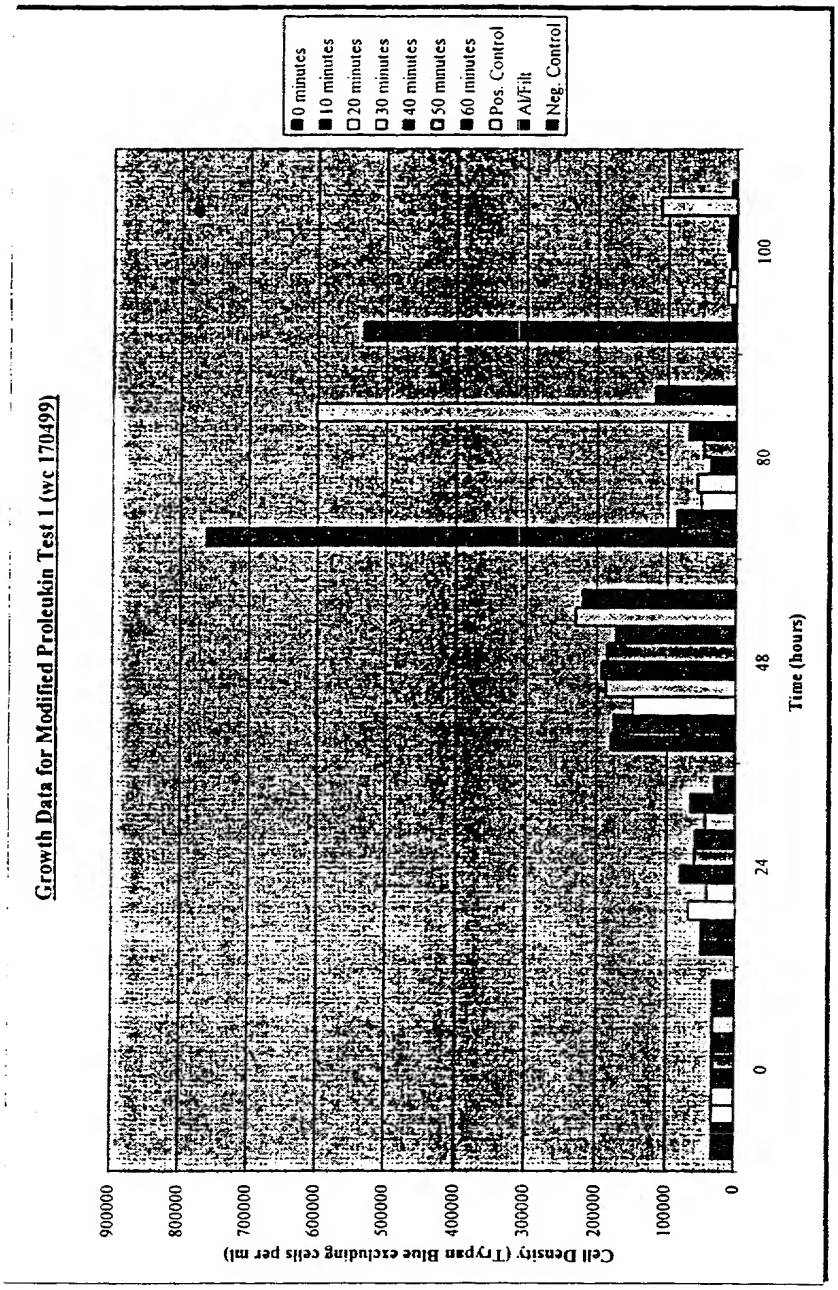

Growth Data for Modified Proleukin Test 1 (wc 170499)
0 minutes
10 minutes
20 minutes
30 minutes
40 minutes
50 minutes
60 minutes
Pos. Control
Al/Filt
Neg. Control
Cell Density (Trypan Blue excluding cells per ml)
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
0
24
48
80
100
Time (hours)

Fig 7

Growth Responses to Proleukin, Native Human Il-2 and Recombinant Human IL-2

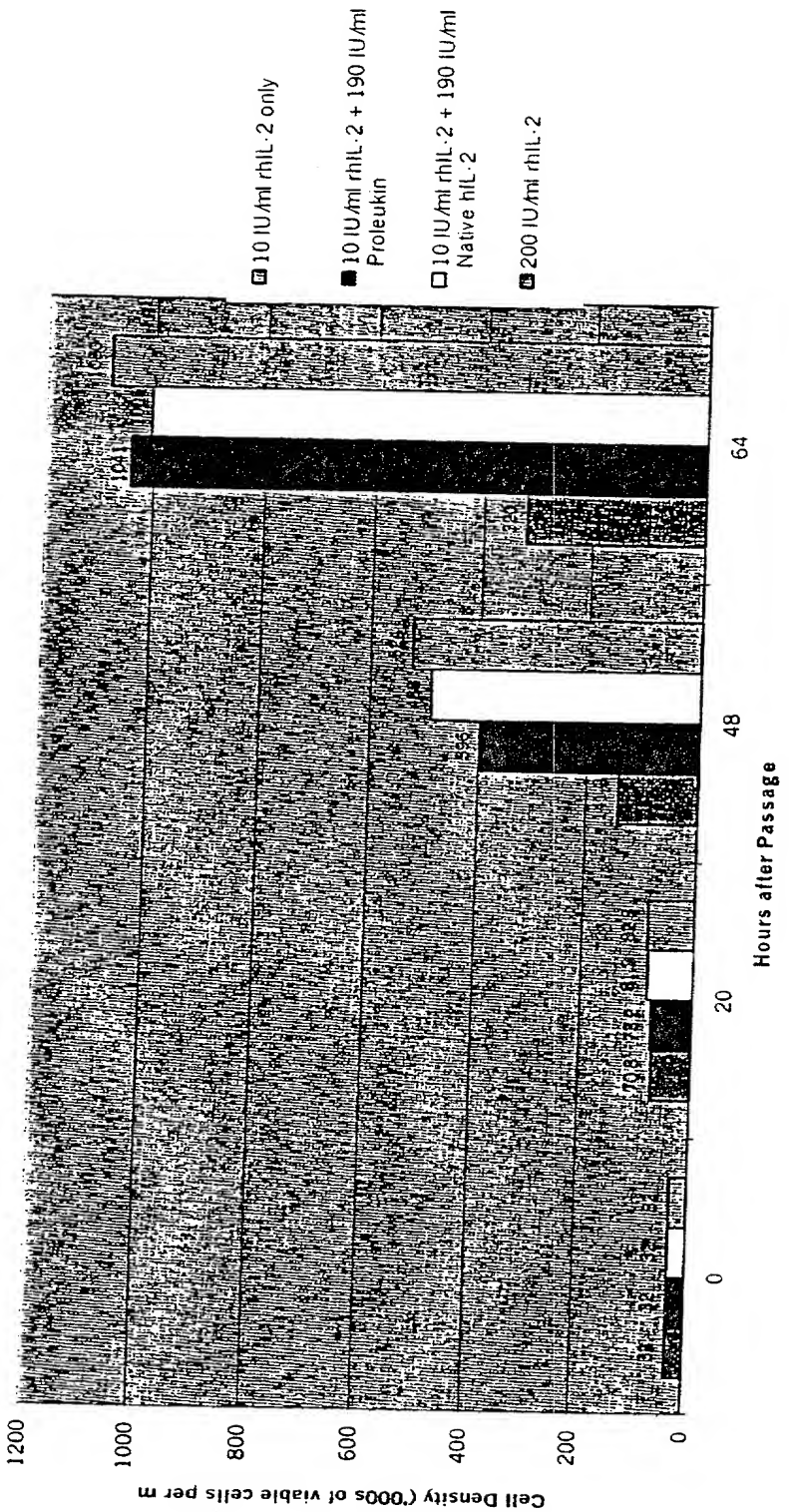


Fig 8

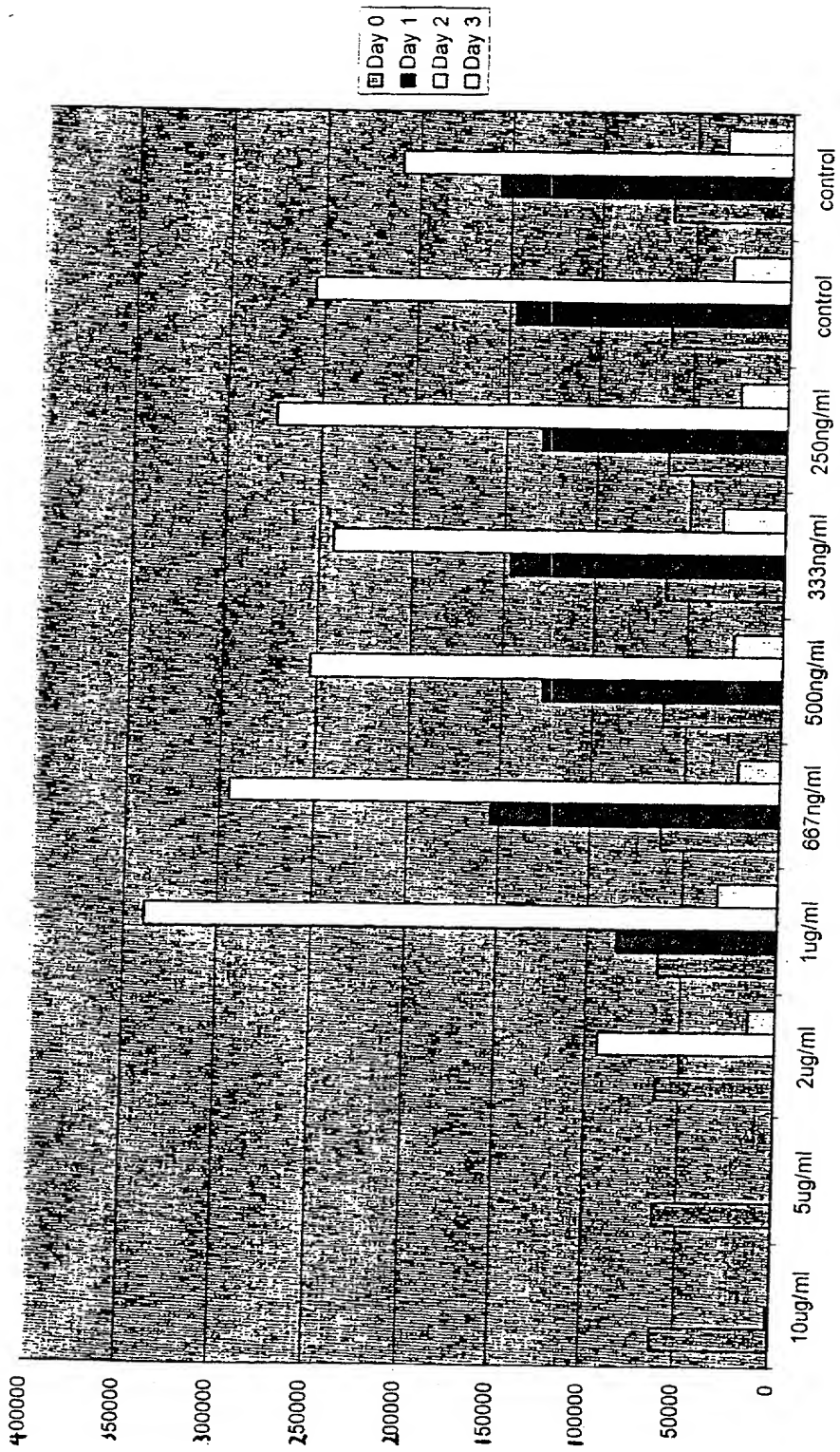

HT-2 Growth response to PEI (1.0ng/ml RhIL-2)
Day 0
Day 1
Day 2
Day 3
400000
350000
300000
250000
200000
150000
100000
50000
0
10ug/ml
5ug/ml
2ug/ml
1ug/ml
667ng/ml
500ng/ml
333ng/ml
250ng/ml
control
control
control
PEI final concentration

Fig 9a

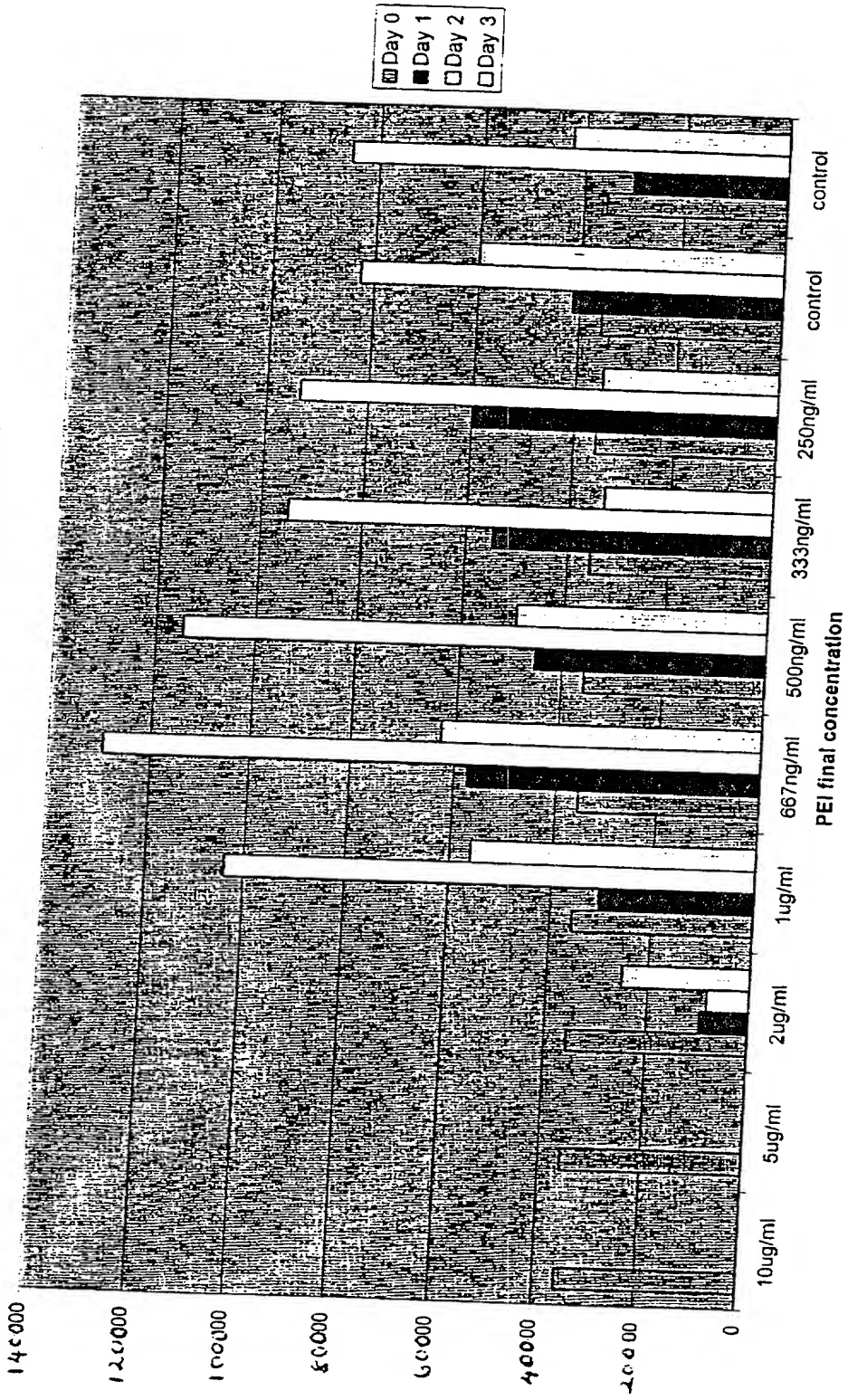

HT-2 Growth response to 25kPEI (0.5ng/mlRhIL-2)
Day 0
Day 1
Day 2
Day 3
140000
120000
100000
80000
60000
40000
20000
0
10ug/ml
5ug/ml
2ug/ml
1ug/ml
667ng/ml
500ng/ml
333ng/ml
250ng/ml
control
control
PEI final concentration

Fig 9b

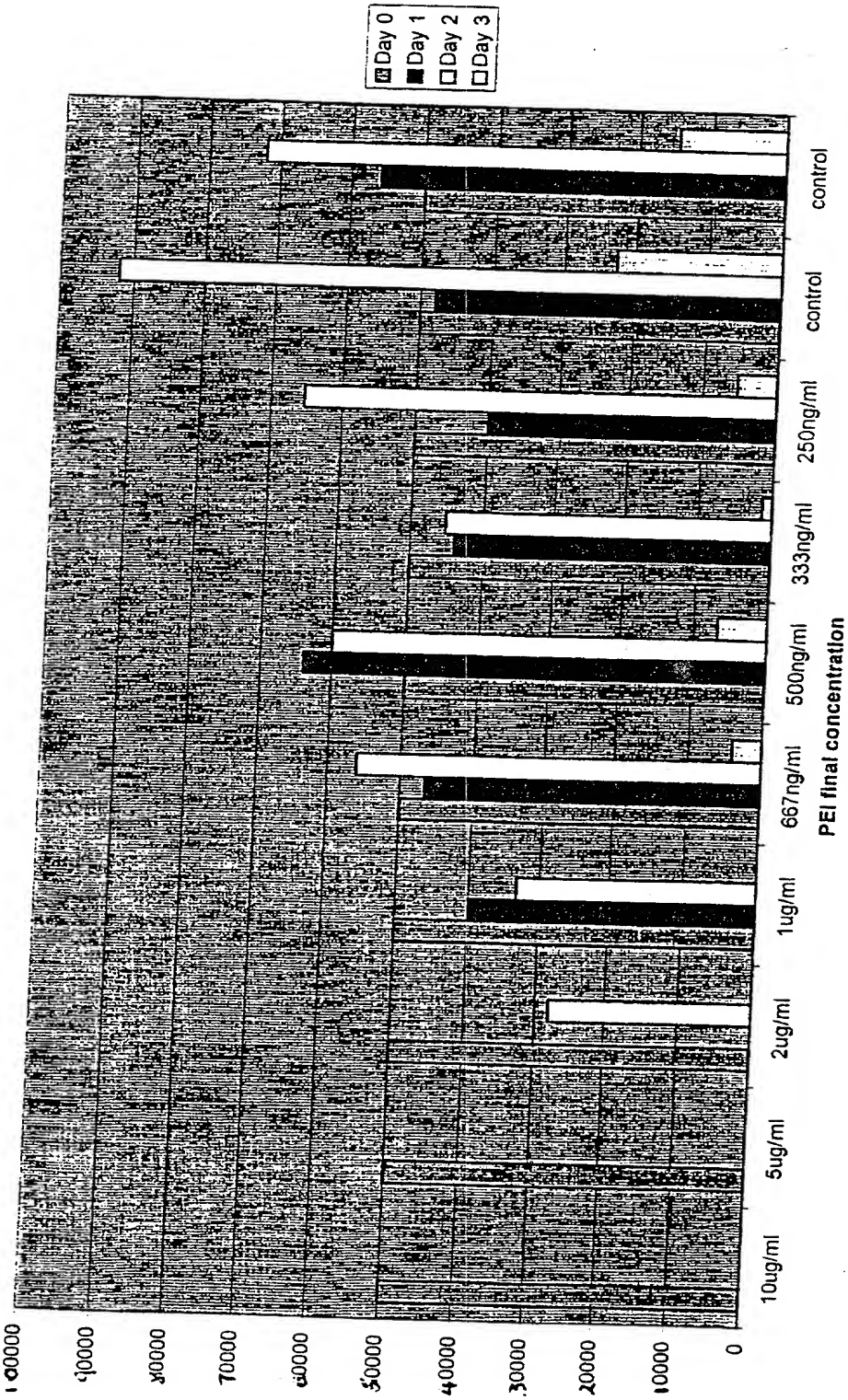

HT-2 Growth Response to 25k PEI (0.2ng/mlRhIL-2)
Day 0
Day 1
Day 2
Day 3
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
10ug/ml
5ug/ml
2ug/ml
1ug/ml
667ng/ml
500ng/ml
333ng/ml
250ng/ml
control
control
control
PEI final concentration

Fig 9c

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled GENE THERAPY PRODUCTS, the specification of which

☐ is attached hereto.

☐ was filed on _____as United States Patent Application No. _____.

☒ was described and claimed in PCT International Application No. PCT/GB00/02014, filed on 5 June 2000, and as amended under PCT Articles 19 on _____ (if applicable).

☐ and was amended on _____ (if applicable).

☐ with amendments through _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R § 1.56. If this is a continuation-in-part application filed under the conditions specified in 35 U.S.C. § 120 which discloses claims and subject matter in addition to that disclosed in the prior copending application, I further acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications(s) on which priority is claimed:

9912807.6	Great Britain	3 June 1999	☒	☐
Number	Country	Day/Month/Year Filed	Yes	No

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

60/137,592	3 June 1999
Application Number	Filing Date

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT international application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the

manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

PCT/GB00/02014	5 June 2000	pending
Application Number	Filing Date	Status: patented, pending abandoned

I hereby appoint the practitioners associated with the customer number provided below to prosecute this application, to file a corresponding international application, and to transact all business in the Patent and Trademark Office connected therewith:

Customer Number 24197
Klarquist Sparkman

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CARLSON, Anne	47,472	RINEHART, Kyle B.	47,027
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JAKUBEK, Joseph T.	34,190	SIEGEL, Susan Alpert	43,121
JONCUS, Stephen J.	44,809	SLATER, Stacey C.	36,011
JONES, Michael D.	41,879	STEPHENS Jr., Donald L.	34,022
KLARQUIST, Kenneth S.	16,445	STUART, John W.	24,540
KLITZKE II, Ramon A.	30,188	VANDENBERG, John D.	31,312
LEIGH, James S.	20,434	WHINSTON, Arthur L.	19,155
MCLEOD, Richard D.	46,921	WIGHT, Stephen A.	37,759
MAURER, Gregory L.	43,781	WINN, Garth A.	33,220
MIRHO, Charles A.	41,199	ZASTROW, Devon J.	P-50,175
NOONAN, William D.	30,878		

I hereby grant the law firm of Klarquist Sparkman, LLP, the power to insert on this Combined Declaration and Power of Attorney any further identification which may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for submitting this document.

Address all telephone calls to Susan Alpert Siegel, Ph.D. at telephone number (503) 226-7391.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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2-00

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WDN/SAS/GLB:clm 11/27/01 Combined Declaration and Power of Attorney

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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